Studies on the muscarinic acetylcholine receptors of the locust

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STUDIES ON THE MUSCARINIC ACETYLCHOLINE RECEPTORS OF THE LOCUST

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"And for ought that I can hitherto discern, there are a thousand phenomena in nature, besides a multitude of accidents relating to the human body, which will scarcely be clearly and satisfactorily made out by them that confine themselves to deduce things from salt, sulphur and mercury, and the other notions peculiar to chymists, without much more notice than they are wont to do, of the motions and figures of the small parts of matter and the other more catholic and fruitful affections of bodies."

Robert Boyle (1661) 'The Sceptical Chymist'.

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-PUBLICATIONS-

Some of the work presented in this thesis has already been published in:

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Duggan, M.J. and Lunt, G.G. (1986) Second messengers linked to the muscarinic acetylcholine receptor in locust (Schistocerca gregaria) ganglia. In Borkovec, A.B. and Gelman, D.B. (editors) Insect neurochemistry and neurophysiology, Humana Press, Clifton, New Jersey.

-SUMMARY-

A muscarinic acetylcholine receptor (mAChR) in the supraoesophageal ganglion of the desert locust Schistocerca gregaria has been studied by the binding of the antagonist [^{3}H]-quinuclidinylbenzilate ([^{3}H]QNB). [^{3}H]QNB binding was inhibited by acetone and ethanol. The mAChR from locust is more susceptible to inhibition by ethanol than the mAChR from rat brain. The profile of temperature dependance for the effect of ethanol is different.

The composition of membrane lipids isolated from the locust supraoesophageal ganglion has been determined. Locust membrane phospholipids lack fatty acids greater than 20 carbon atoms in length. Locust membranes have a lower viscosity, determined by fluorescence polarization, than that reported for rat brain membranes.

Phosphatidylinositol has a higher turnover rate than the other phospholipids in the locust ganglion. Incorporation of [^{3}H]inositol is increased by the cholinergic agonist carbamoylcholine and this effect is blocked by the muscarinic antagonist atropine.

The production of adenosine-3',5'-monophosphate, in homogenates of the cerebral ganglion was inhibited by carbamoylcholine but not in the presence of atropine.

The evidence, from the involvement of mAChRs in two second messenger systems, that there may be more than one class of mAChR is supported by the inhibition of [^{3}H]QNB by the selective ligands, pirenzepine, hexahydrosiladifenidol and AF-DX 116. These ligands give inhibition curves that do not fit models with one class of binding site.

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CHAPTER 1.

INTRODUCTION

-Background-

1.0

There are about one million species of insects known to man. Not only do they outnumber the 47,000 species of the entire phylum Chordata but they also have an immense economic impact on mankind. Cramer (1967) calculated that a conservative estimate of the loss of the potential agricultural production of the world was 14% from insect pests alone. This figure did not include losses in pastoral farming or the effect of the diseases for which insects serve as the vector, for example sleeping sickness and malaria.

To combat these competitors man uses a variety of chemical insecticides, most of which are neurotoxins. However our knowledge of insect neuroscience has been so limited that the modes of action of these compounds are not properly established. Indeed basic insecticide research is often carried out in vertebrate tissues, which are much better characterized, despite some obvious differences between the nervous systems of vertebrates and insects. This same lack of knowledge has meant that the development of novel insecticides, needed to overcome the resistance that insects are developing to those already in use, has been by near-random "synthesise and screen" methods. Casida (1979) has estimated that by this process it takes about 50 man-years of chemistry to produce a viable insecticide.

Following the lead of the pharmaceutical industry some agrochemical concerns are now looking to shorten this time by the rational design of compounds to attack defined target sites. This has lead to an increased effort in basic insect neurobiology and particularly in insect neurochemistry.

-The CNS of the Locust-

1.1

In insect embryos the body is segmented and each segment contains a bilaterally symmetrical ganglion. These ganglia fuse, to some degree, during development to form the central nervous system (CNS). The most anterior ganglion in the insect after hatching is the supraoesophageal ganglion (also known as the cerebral ganglion or brain), it includes ganglia fused together with the primitive, presegmental archecerebrum. From this the circumoesophageal connectives run, one on each side of the oesophagus to the suboesophageal ganglion. The suboesophageal ganglion is the first ganglion in the ventral nerve cord, which is a chain of linked ganglia running the length of the body.

1.1.1

The ganglia are all of the same general

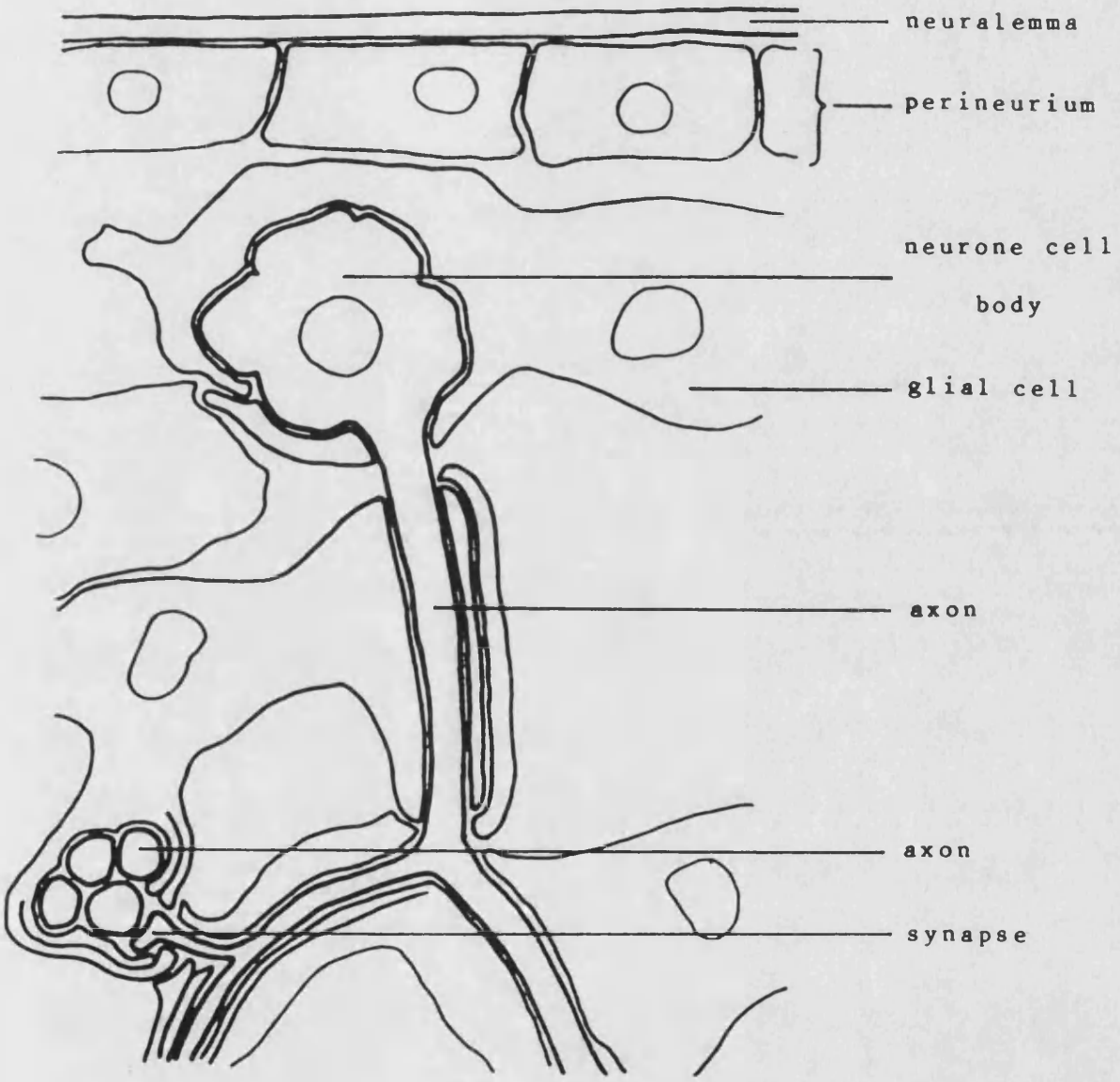

Figure 1. Representation of a section through an insect ganglion.

structure (see Fig. 1). The mass is surrounded by an acellular structure called the neural lamella over a cellular layer, the perineurium. Under the perineurium are found the cell bodies of the neurones, supported by glial cells, which are peripheral to the neuropile, a complex of axons and dendrites which is where all synapses are believed to occur. In insects the axons are not myelinated and there are few glia in the neuropile. This localisation of function and the lack of axo-somatic synapses is in contrast with the vertebrates where cell bodies do receive synaptic connections.

1.1.2

This thesis is concerned with some aspects of the neurochemistry of the supraoesophageal ganglion.

Structurally the supraoesophageal ganglion is composed of 3 main parts, the protocerebrum, deutocerebrum and tritocerebrum (see Fig. 2). The protocerebrum is the largest of these three parts and may be the seat of behavioural organisation (Chapman, 1982). The deutocerebrum contains the antennal lobes, containing the cell bodies of the sensory neurons, and the tritocerebrum is the point of origin for the connectives to the suboesophageal and frontal ganglia.

This is a more complex structure than the ganglia of the ventral nerve cord as it contains several areas of neuropile where the input from the sensory organs located on the head is processed (see Fig. 3).

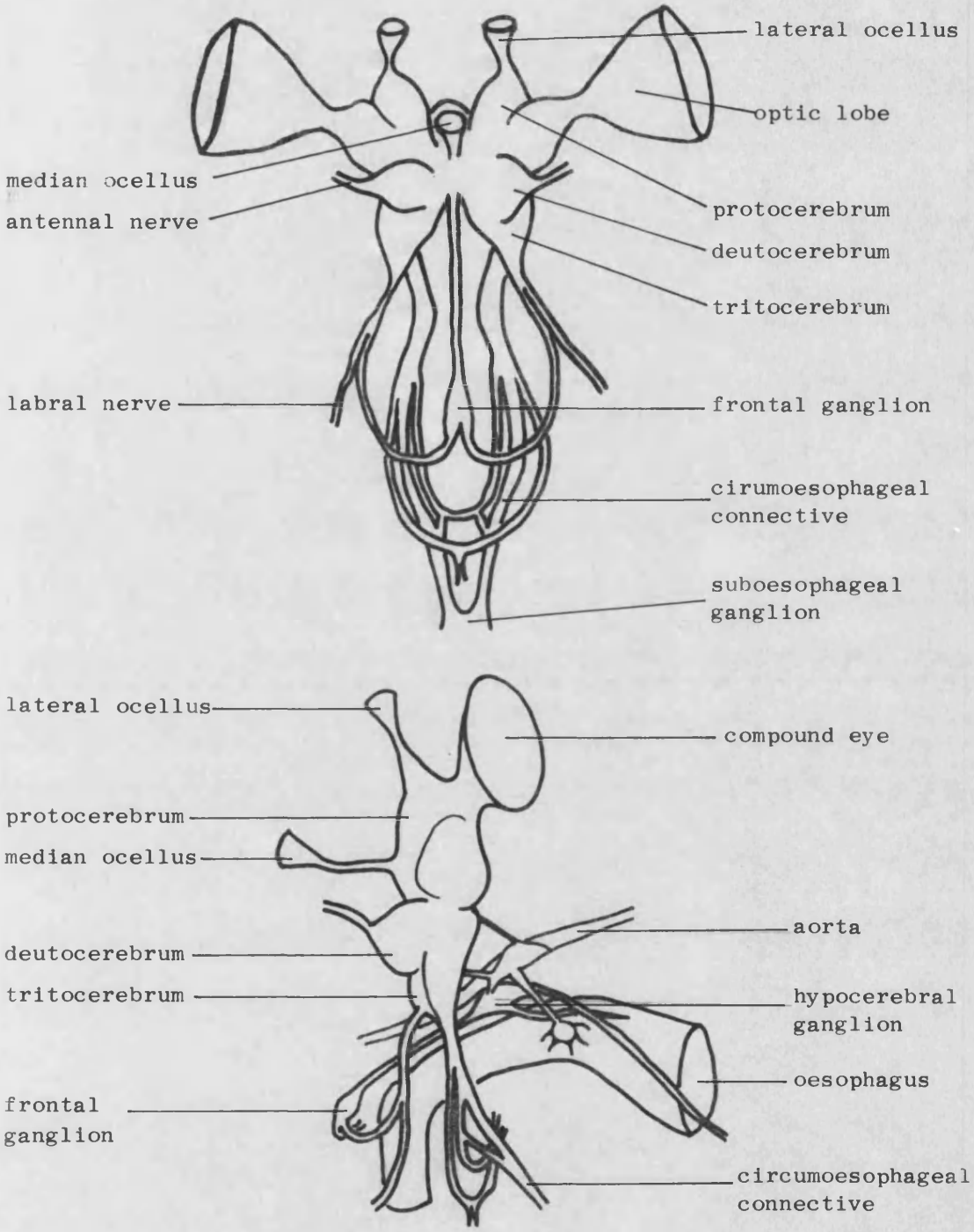

Figure 2. Anterior and lateral views of the brain and stomatogastric nervous system of the locust (after Albrecht, 1953)

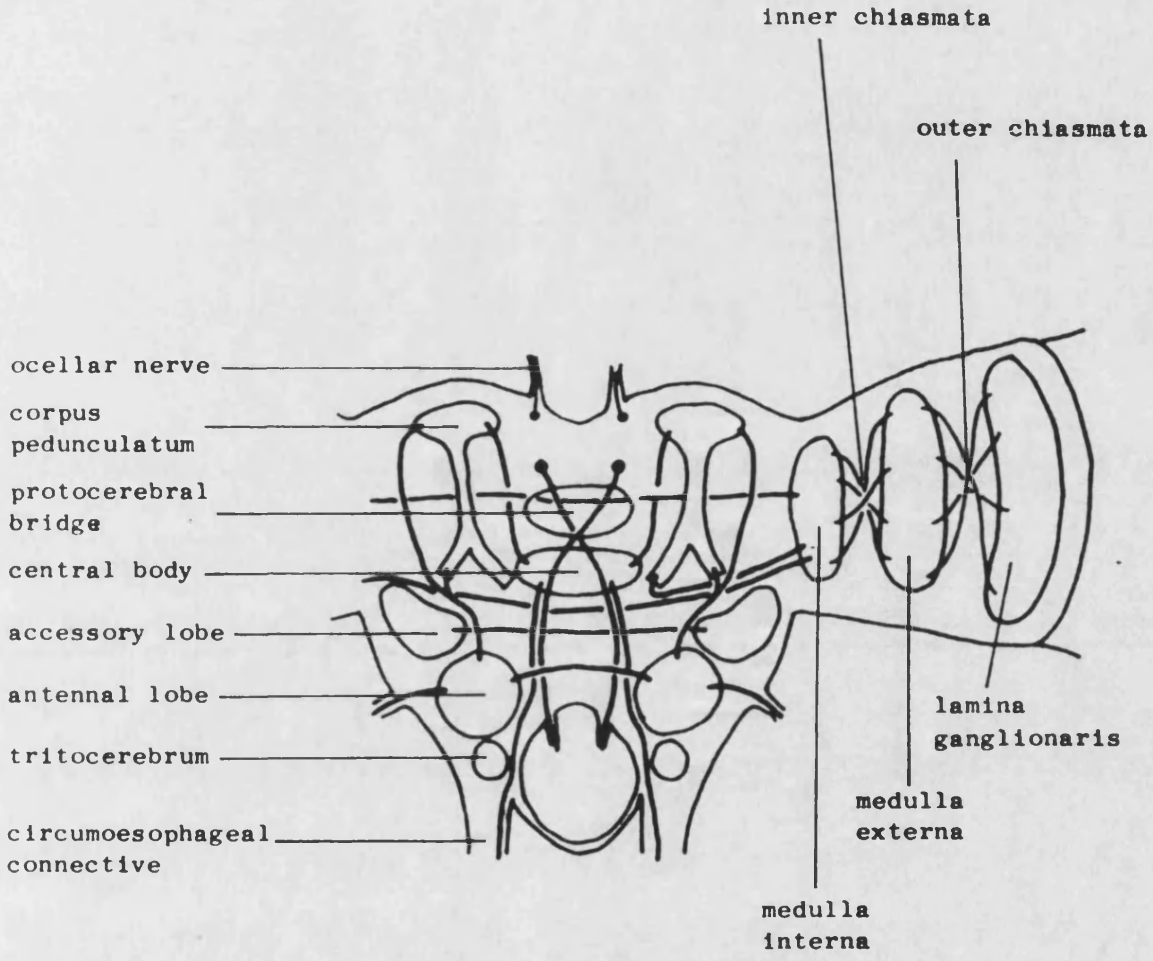

Figure 3. Diagram of the brain of Locusta migratoria showing the more important areas of neuropile and a few of the main connections between these areas.

-Cholinergic Transmission-

1.2

Neurones impinge on other cells, including other neurones, at the synapse. The synapse is a specialised structure at which the ending of an axon approaches, in the case of an insect neurone-neurone synapse, a part of the arborisation of another neurone.

The structure and some of the functions of a cholinergic synapse is shown (Fig. 4). The presynaptic action potential leads to depolarisation of the terminal and the ensuing influx of Ca^{2+} ions causes release of acetylcholine (ACh) stored in the synaptic vesicles. The ACh then diffuses across the synaptic cleft and interacts with the receptor molecules situated in the membrane of the postsynaptic cell which transform this signal into the cellular response, this interaction takes the form of a reversible binding. The ACh also interacts with autoreceptors on the presynaptic terminal. There are both muscarinic and nicotinic autoreceptors in vertebrates. Muscarinic autoreceptors have been shown to form part of a negative feedback loop which may act to terminate the synaptic event (Polak, 1971 and Nordström and Bartfai, 1980). The nicotinic autoreceptors, however, seem to increase ACh release (Moss and Wonnacott, 1985).

The ACh diffuses away from the receptors and is prevented from accumulating in the synaptic cleft by

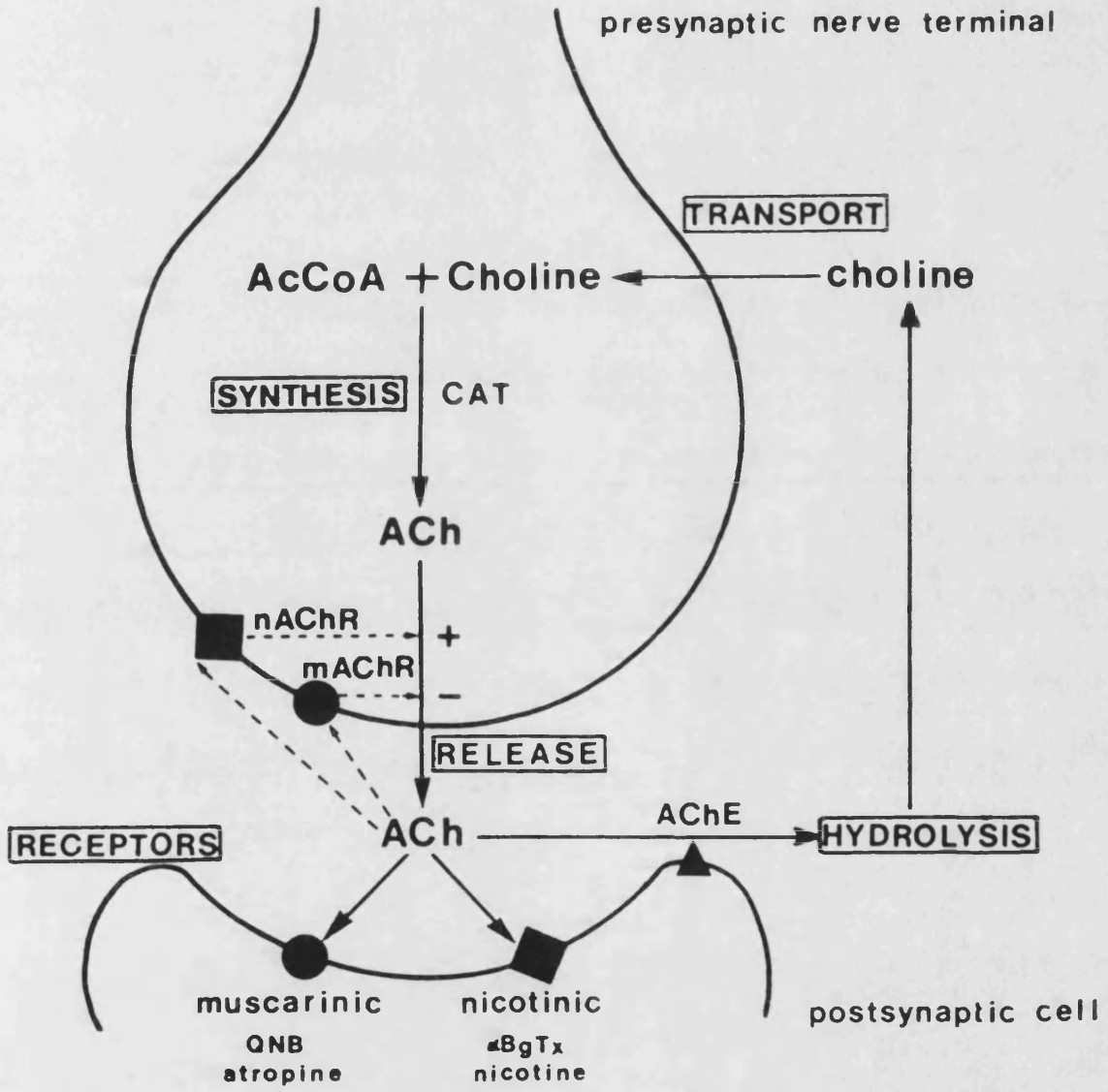

Figure 4. Model of a cholinergic synapse.

the enzyme acetylcholinesterase. The products of the hydrolysis of ACh are acetate and choline, both of which are taken up into the presynaptic nerve terminals by an active transport system and may be reincorporated into acetylcholine.

1.3 -The Importance of The Membrane-

The receptors for ACh, and for other transmitters, are transmembrane proteins, spanning the lipid bilayer and in intimate contact with it. This means that the function of the receptors, and thus of the nervous system, may be dependent on the state of the whole membrane.

Current thoughts on the structure of the biological membrane are largely influenced by the fluid mosaic model of Singer and Nicolson (1972). They proposed that the lipid bilayer is largely fluid. This "sea" of lipid supports "icebergs" of protein which are immersed in it to varying degrees. Both the lipid and protein components are free to move in the plane of the membrane. However it has been shown that there are some constraints on the movement of the components of the membrane (for review see Helmreich and Elson, 1984).

Proteins may be constrained by interaction with cytoskeletal elements; Rousselet et al. (1981) have presented evidence that the nAChR in the electric organ from Torpedo electric organ are held together in a rigid array by a non-membrane protein.

Lipids, on the other hand, may form close associations with membrane proteins which result in a ring of annular lipid around the protein, maintaining it in a constant lipid environment. Two components, each of different fluidity, have been observed in several protein rich membranes, including for example the mitochondrial membrane containing cytochrome oxidase (Jost et al., 1973a). This was interpreted as evidence for two regions of lipid bilayer, corresponding to that in contact with proteins and the rest in a free lipid-only environment. It was proposed that the former of these was a rigid or immobilised layer of annular lipid (Jost et al., 1973b). However this interpretation has been challenged; Chapman et al. (1979) suggest that there are other possible explanations for the observations made and review evidence which cannot be reconciled with the concept of a fixed lipid annulus. In particular, data from deuterium nuclear magnetic resonance studies (Kang et al., 1979) which measure slower events (10^5 s^{-1}) than the electron spin resonance studies of Jost et al. (1973a,b) (10^8 s^{-1}), do not show two different lipid components. Chapman et al. (1979) suggest that this indicates that any lipid in

association with protein is therefore not tightly bound but free to exchange with other lipid in the membrane.

The characteristics of the plasma membrane are a resultant of the interactions of the several lipid constituents, which form about 40% of the dry weight of most membranes, and the proteins asociated with the membrane, which comprise the remaining 60%. The lipid composition of vertebrate nerve ending membranes has been determined both in the proportion of the various lipid classes and the fatty acid composition within each class of phospholipid (Breckenridge et al., 1972). In insects the classes of lipid at nerve endings has been determined (Breer and Jeserich, 1984), but work on fatty acid compositions has been largely limited to experiments on whole insects (for example see Hanson et al., 1985).

1.3.1

Compounds such as aliphatic alcohols and some anaesthetics are known to change membrane properties. The membrane fluidity is known to increase under their influence (Papahadjopoulos et al., 1975) and this is thought to occur by disordering and fluidizing the hydrophobic core of the lipid bilayer (Chin and Goldstein, 1981).

The effect of ethanol on the fluidity of vertebrate synaptosomal membranes has been studied (Chin and Goldstein, 1977; Harris and Schroeder, 1981) and

they have been found to be more sensitive to membrane disordering compounds when they are intact than the lipid isolated component is when reconstituted (Harris and Schroeder, 1981).

The changes induced in brain membrane fluidity have been found to correlate with hypnotic potencies in mice for a series of short-chain alcohols (Lyon et al., 1981).

There is evidence that membrane disordering compounds have a direct effect on receptor function. Studies in vitro on neuromuscular junction properties have found that octanol changes the kinetics of ACh induced minature endplate potentials, by reducing the time constant of the decay phase (Gage et al., 1974), as do free fatty acids (Andreasen and McNamee, 1980). El-Fakahany et al. (1983) observed that alcohols change the affinity of the nAChR ion channel for ligands such as histrionicotoxin which is an ion channel blocker.

Aguilar et al. (1980) found that local anaesthetics which are known to have membrane effects (Jain et al., 1975; Papahadjopoulos et al., 1975) inhibited [^{3}H]QNB binding to central muscarinic receptors, acting at a site other than that where QNB binds. El-Fakahany and Richelson (1981) found that other anaesthetics blocked muscarinically stimulated cGMP formation as well as [^{3}H]QNB binding, in a noncompetetive fashion, and were more effective at supressing the cGMP system, which involves a

membrane-protein interaction. Ethanol has been shown to change the characteristics of binding to brain opiate receptors both enhancement and inhibition of binding being observed depending on the concentration used (Tabakoff and Hoffman, 1983).

It would seem that the these compounds have their effect by interfering with the interactions between receptor proteins and their lipid enviroment. There is also evidence that insecticides may influence the characteristics of membranes (Marquis, 1985). In rat erythrocytes Domenech et al. (1977) found that concentrations of the anticholinesterase organophosphates which do not inhibit the enzyme changed the Hill coefficient of the enzyme through a membrane fluidity effect.

It has been reported that specific classes of membrane lipid affect receptor function for nAChRs (Zabrecky and Raftery, 1985) opiate receptors (Abood and Takeda, 1976) and mAChRs (Aronstam et al., 1977). Zabrecky and Raftery (1985) altered the lipid composition of receptor rich membranes from Torpedo californica by fusing them with liposomes of a known composition. They found that a high cholesterol concentration was important for carbamoylcholine (carbachol) activation of the ion channels, and that the proportion of the different phospholipid classes had an effect on the ion flux through the channel.

Abood and Takeda (1976) found that

phosphatidylserine (PS) enhanced stereospecific binding to opiate receptors when incorporated into the neural membranes, whereas other phospholipids had deleterious effects or no effect at all. Aronstam et al. (1977) found that phospholipase treatment inhibited [^{3}H]QNB binding, but this could be partly restored by the subsequent addition of exogenous phospholipids, of which PS was the most effective.

It has not been shown whether these effects are due to phospholipid binding to the receptor molecules or to membrane fluidity effects. Cholesterol is known to increase membrane viscosity (Chin and Goldstein, 1981) and it may be that this is its principle mode of action. The possibility of low affinity binding sites on the protein cannot, however, be ruled out.

The studies on phospholipid effects are difficult to interpret as the phospholipids used were mostly crude preparations without a defined fatty acid composition and therefore had an unknown effect on membrane properties. The exception is the study of Aronstam et al. (1977), they did use some synthetic phospholipids, but beyond observing that the fatty acid composition has some effect on the phospholipids' efficacy in modifying receptor function did not study this aspect further.

-Muscarinic Acetylcholine Receptors-

1.4

In vertebrates acetylcholine is a neurotransmitter with functions in both the central and peripheral nervous systems. Like other neurotransmitters the effects of acetylcholine are mediated by cell surface receptors, acetylcholine receptors (AChR).

There are two major types of AChR, nicotinic (nAChR) and muscarinic (mAChR), which differ in the responses they evoke, in their location and in their pharmacology (Dale, 1914). The nAChR is found classically at the neuromuscular junction, but also in the CNS, and gives rise to fast stimulatory responses. The mAChR is found in the CNS and smooth muscle, for example that of the heart and gut, and in different tissues may lead to either stimulatory or inhibitory reponses, which tend to be slower in onset and longer in duration.

In the vertebrate CNS there are known to be many cholinergic pathways, and mAChRs have been associated with important functions in cognitive function and memory (Hoss and Ellis, 1985).

1.4.1

Much of the work that has been performed to characterize the mAChR in the vertebrates has been based

on the binding of radiolabeled ligands, particularly antagonists such as quinuclidinylbenzilate (QNB) and N-methylscopolamine. For a long time the work of many laboratories suggested that there was a single homogeneous class of mAChR in all the tissues investigated, and that the properties measured agreed with the responses to antagonists in physiological assays (Birdsall et al., 1980; Snyder et al.,1975). However closer examination of the inhibition of the binding of these compounds by agonists showed concentration-dependence curves which deviated from those expected for a homogeneous population of sites. The inhibition curves are flattened and the Hill coefficients are significantly less than one (Birdsall et al., 1978, 1980). Parallel investigations that used labelled agonists lead to the formation of a model which postulated that there are multiple subpopulations of mAChRs that are discerned by agonists but not by antagonists (Birdsall et al., 1978).

There has been some doubt regarding the number of binding sites for agonists. A two site model was not capable of explaining the variations between different brain regions, whereas a three site model was sufficient (Ellis and Hoss, 1980). These data agreed with the curve fitting experiments of Birdsall et al. (1980), who found that a three site model significantly improved the fit over a two site model. These data must be treated with some caution as the comparison of models of great

complexity, such as those with three sites or those with two sites, one of which shows cooperativity is not easy (Sokolovsky et al., 1983).

1.4.2

There are now available some selective or 'non-classical' antagonists. Hammer et al. (1980) showed that the antagonist pirenzepine inhibited the binding of the classical antagonist N-methylscopolamine with potencies that varied between different tissues and had Hill coefficients significantly less than unity. This compound along with the non-classical agonist McN-A-343 was used to distinguish between two subclasses of mAChR, M_1 and M_2 (Goyal and Rattan, 1978; Hammer and Giachetti, 1982). Receptors of the M_1 type were stimulated by McN-A-343 in functional assays and had a high affinity for pirenzepine, whereas M_2 receptors were not stimulated by McN-A-343 and had a lower affinity for pirenzepine.

Since then two more non-classical antagonists have been reported, AF-DX 116, a pirenzepine derivative (Giachetti et al., 1986; Hammer et al., 1986) and hexahydro-siladifenidol (Mutschler and Lambrecht, 1984). These two compounds are selective for the M_2 subclass of mAChR but show variable affinities for different tissues and have been used, along with pirenzepine, to define a three subclass model for the mAChR (Birdsall et al., in press). This would seem to agree with the three site

model for agonist binding to mAChRs, however Watson et al. (1986) have reported that there is some remaining heterogeneity of agonist binding within the M_1, high pirenzepine affinity, subclass.

There has been much discussion as to whether the different sites observed are interconvertable or not. Berrie et al. (1986) have observed that solublized receptors maintain full heterogeneity of ligand binding whereas Baron et al. (1985) have noted a conversion by transition metal ions to a single class of binding site for agonists. Such contradictory results have lead to a debate about whether these different subclasses represent different conformational states of the same protein or proteins with different sequence. The recent publications by Kubo et al. (1986a,b) of the amino acid sequences for two isoforms of the porcine mAChR have apparently resolved this debate. The two isoforms are highly homologous for much of their sequence but have a region of great heterogeneity which, it is proposed, may be the site of their interactions with the effector mechanisms. Some speculation may remain as to whether any more molecular forms of the receptor exist.

-Second Messengers Linked to mAChR-

1.5

Unlike the nAChR which has its effect through the opening of an integral ion channel, the mAChR may be linked to several different effector mechanisms. These include four different membrane ion conductance changes (North, 1986), in different tissues potassium conductances can be either increased or decreased, a sodium conductance can be increased and a calcium conductance can be decreased. Second messenger effects include stimulation of guanylate cyclase (Hanley and Iversen, 1978), inhibition of adenylate cyclase (Olianas et al., 1983) and stimulation of phosphatidylinositol (PI) turnover (Fisher, 1986).

There is some uncertainty over how the ion channels and guanylate cyclase are linked to the receptor, for example although some potassium channels seem to be linked intimately with the mAChR (Sakmann et al., 1983) other ionic responses show a latency which far exceeds the normal synaptic delay incurred in nicotinic transmission (Brown et al., 1986), this suggests the possible involvement of a second messenger responsible for the activation of the electrophysiological event. It has been shown that the second messengers from PI metabolism produced in response to bradykinin can change the membrane potassium conductance of a neuronal cell (Higashida and Brown,

1986).

The two mechanisms that have been most studied are the downregulation of adenylate cyclase and the turnover of PI.

-Adenylate cyclase regulation-

1.5.1

Adenylate cyclase is a membrane bound enzyme which catalyzes the conversion of adenosine-5'-triphosphate (ATP) to adenosine-3',5'-monophosphate (cAMP). cAMP is an important second messenger in the CNS (for review see Drummond, 1983) and acts to stimulate the activity of protein kinases. Different neurotransmitters and hormones can act either to stimulate or inhibit adenylate cyclase (Drummond, 1983). The regulatory effect is mediated by the interaction of the receptor with the guanine nucleotide-binding proteins (N-proteins) that regulate adenylate cyclase (see Fig. 5).

N-proteins are composed of three subunits, α, β and γ. The β and γ subunits are common to both the stimulatory (N_s) and inhibitory (N_i) nucleotide-binding proteins. The α subunit however is different and it is this that binds the GTP and has the GTPase activity.

The model for N-protein action has been

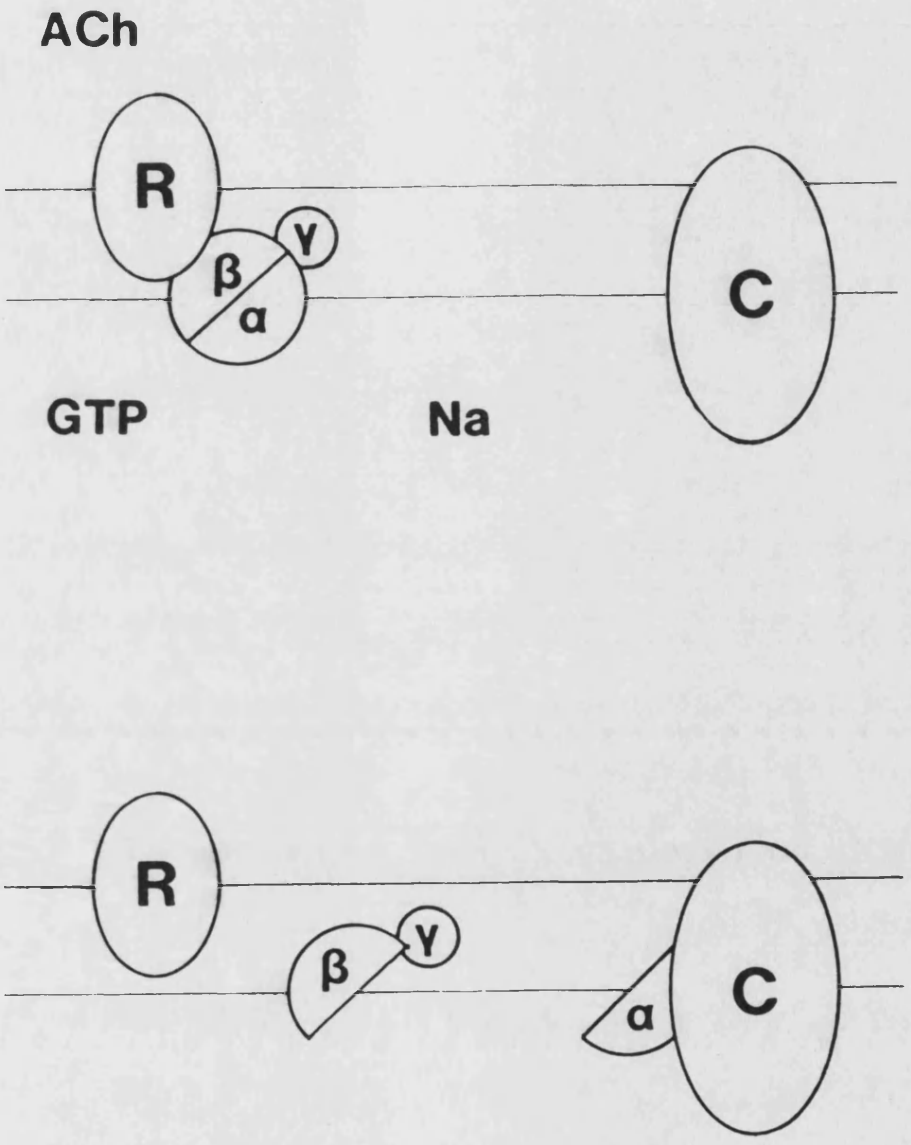

Figure 5. Model proposed for the mechanism of action of the inhibition of adenylate cyclase activity by acetylcholine.

In the presence of acetylcholine, sodium and GTP the muscarinic receptor (R) causes the dissociation of the N_i protein complex allowing the α-subunit to interact with the adenylate cyclase (C) and cause a reduction in its activity.

proposed by Hildebrandt et al. (1984) and Katada et al. (1984). The occupancy of the receptor by an agonist facilitates the binding of GTP to N_s and the consequent dissociation of the α subunit. This allows (in the case of the N_s) formation of active α_s-GTP complexes which stimulate the catalytic subunit of the cyclase. This stimulation process is under negative control by the GTPase activity, which catalyzes the hydrolysis of α_s-GTP to α_s-GDP, thereby turning off the stimulation of the cyclase by the active α_s-GTP complex. The α subunit dissociates from the adenylate cyclase and reassociates with the other subunits to form N_s. The model is the same for inhibitory responses except that it has been suggested that the inhibition may be enhanced if the free β/γ complex associates with α_s subunits preventing their access to the adenylate cyclase. It has been shown that the inhibitory coupling of muscarinic receptors to adenylate cyclase occurs via N_i (Onali et al., 1983) and is sodium dependent.

-Phosphatidylinositol metabolism-

1.5.2

PI is a membrane phospholipid which is found in all eukaryotes and has been reported to have many different and important functions (for review see Berridge, 1984).

The first report of muscarinic stimulation of PI metabolism in the brain was made by Hokin and Hokin (1955), who followed the incorporation of ^{32}P into PI and phosphatidic acid (PA) as a measure of their turnover, and found that this was enhanced by mAChR stimulation. The labile PI pool has been shown to be located in plasma membrane associated with nerve endings (Lunt and Pickard, 1975) and in neurons rather than glia (Fisher et al., 1981).

PI is also unique among phospholipids in that it can be further phosphorylated to give phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-diphosphate (PIP_2). These lipids have been found to form a high proportion of phosphoinositides in neural tissues (Hauser and Eichberg, 1973) and are largely present in the plasma membrane (Eichberg and Hauser, 1973).

The importance of these polyphosphoinositides and particularly of PIP_2 was shown by Michell et al. (1981) who showed that, in hepatocytes stimulated by vasopressin, decreases in PIP_2 precede changes in PI. The primary receptor stimulated event is then the hydrolysis of PIP_2 to yield sn-1,2-diacyglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). This has been confirmed by (among others) Berridge (1983) and Berridge et al (1984), working with the salivary gland of the blowfly (Calliphora), who have shown that IP_3 is produced immediately on stimulation whereas the

formation of IP, the expected product for the hydrolysis of PI, and the physiological response are delayed. The subsequent model is portrayed in Fig. 6.

The two products of the hydrolysis of PIP_2 are both thought to act as second messengers. IP_3 has been shown to release Ca^{2+} from intracellular stores, thought to be part of the endoplasmic reticulum (for review see Berridge and Irvine, 1984).

DAG is thought to remain in the membrane and has been shown to activate protein kinase C. Protein kinase C is not activated by Ca^{2+}-calmodulin or cAMP, nor does it, in general, catalyse the phosphorylation of the same substrate proteins as those that are phosphorylated by other kinases (Berridge, 1984).

The hydrolysis of PIP_2 is catalyzed by a specific phospholipase-C (referred to by many workers as a phosphodiesterase), how this is linked to the receptor has not been fully demonstrated but there is evidence that indicates that an N-protein may be involved which seems to be neither N_i nor N_s (Cockcroft and Gomperts, 1985; Taylor and Meritt, 1986).

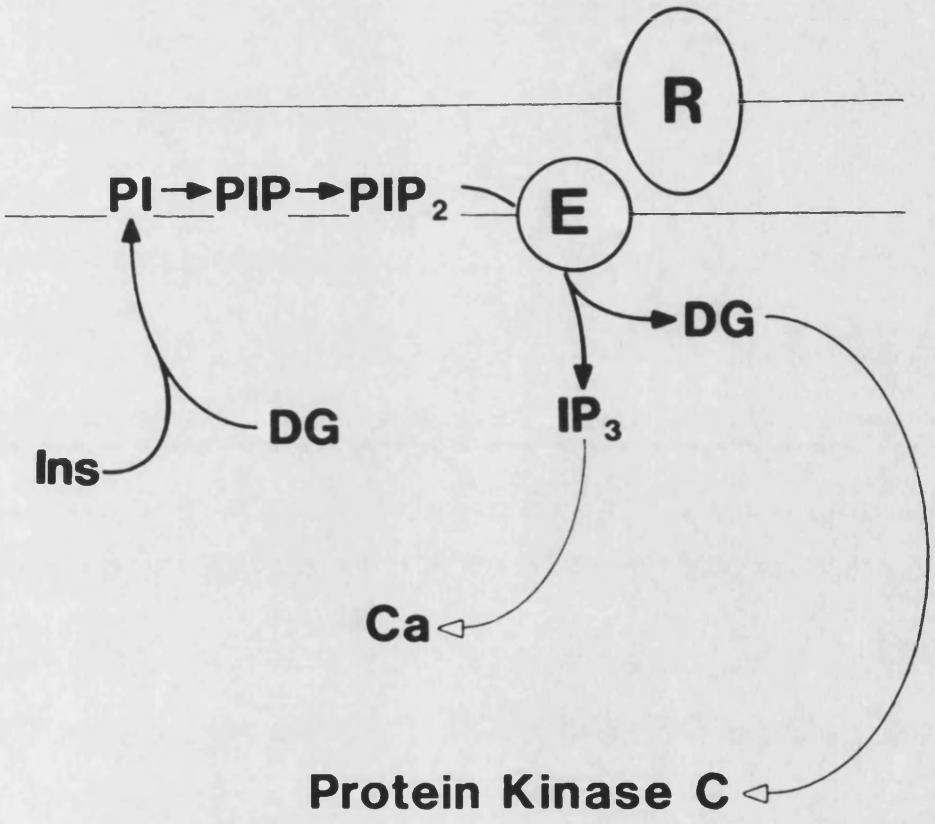

Figure 6. Model proposed for the propagation and action of the second messengers derived from PtdIns.

-AChR in the Insect CNS-

1.6

Acetylcholine (ACh) is known to be an important neurotransmitter in the insect CNS (see Gerschenfeld, 1973; Sattelle, 1980; 1985 for reviews). The essential components of a cholinergic system have been found in several insect species, in locusts Breer (1981) has shown that levels of ACh and the enzymes of its metabolism, acetylcholinesterase and choline acetyltransferase, are much higher in the cerebral ganglion of the locust than in the cerebral cortex of the mouse. Breer (1981) also demonstrated the presence of a high affinity choline uptake system in a preparation of isolated nerve terminals from the cerebral ganglion of the locust.

-Mixed pharmacology AChR-

1.6.1

Radioligand binding assays have been used to identify putative acetylcholine receptors (AChR). This technique was first used by Eldefrawi and O'Brien (1970) who used [^{3}H]-muscarone to probe for AChR in aqueous extracts of house fly (Musca domestica) heads. The binding site appeared to satisfy the criteria for a receptor binding site, and was inhibited both by ligands

that in vertebrates are designated as muscarinic in nature and by those which are thought of as nicotinic.

Following these studies the same extract was found to bind radiolabelled nicotine, decamethonium, dimethyl d-tubocurarine and atropine (Eldefrawi et al., 1971). The pharmacologies of the muscarone and nicotine binding sites were found to be similar (Eldefrawi et al., 1970) and so they were thought to be the same binding site.

Further work by Donnellan et al. (1975) showed that proteolipids extracted from a high speed supernatant from Musca domestica heads bound radiolabelled decamethonium, tubocurarine and ACh. When these proteins were eluted from Sephadex LH-20 columns with chloroform: methanol the binding sites for the three radioligands were eluted with the same retention time. This binding site did not bind [^{3}H]acetyl-αbungarotoxin, the classical ligand for vertebrate peripheral nAChR, nor was the binding of the other radiolabels inhibited by αbungarotoxin. This established that this binding protein had a unique pharmacology and resembled a putative AChR of a novel type.

This putative receptor has been further characterised as a glycoprotein complex running on gel permeation columns with a molecular weight of approximately 350 K and an isoelectric point between 4.8 and 5.0 (for review see Sattelle, 1980).

This binding site is unusual both for its novel pharmacology and because it seems to be soluble in aqueous media. Since the time that [^{3}H]decamethonium, the most specific ligand for this putative receptor, ceased to be commercially available the study of this binding protein has lapsed. The ready solublisation of the protein without the detergents usually required for receptors may have cast some doubt on its true status as a neurotransmitter receptor. At one time it seemed that a mixed pharmacology AChR might be an evolutionarily primitive class of AChR, resembling an ancestral AChR from which the nAChR and mAChR had diverged evolutionarily. This now seems to be unlikely since the publication of amino acid sequences for vertebrate nAChR (Noda et al., 1983) and mAChR (Kubo et al., 1986a) which do not show any significant homology. This suggests that they represent an example of separate or even convergent evolution of different lineages, but responding to the same physiological stimulus.

-Nicotinic AChR-

1.6.2

The mixed pharmacology AChR was shown not to bind the nicotinic antagonist αbungarotoxin (Mansour et al., 1977). However the iodinated toxin (^{125}I-αBgTx) was shown to bind to a site in the particulate fraction of

heads from the fruit fly (Drosophila melanogaster) but not to the supernatant where the mixed pharmacology AChR has been found (Schmidt-Nielsen et al., 1977). This indicated that there was also a nAChR in the insect nervous system similar to that found in the vertebrate neuromuscular junction.

The pharmacology of a [^{125}I]αBgTx binding site has been investigated in several insect species, and correlated with the pharmacology of ACh gated ion channels in the cockroach CNS (Sattelle, 1985). These studies seem to confirm that it is a nAChR similar to that found at the vertebrate neuromuscular junction and so the purification methods applied to the nAChRs from vertebrates have been applied to insect tissues. The purification techniques involve affinity chromatography using snake venom toxins linked to gel supports. Several groups have reported degrees of purification of the nAChR from insects but subsequent denaturing gel electrophoresis has neither given the same pattern of subunits found in vertebrate tissues nor a consistent pattern among the insect species (for reviews see Sattelle, 1985; Lunt, 1986). For example Breer et al. (1984) reported that the αbungarotoxin-binding component from cerebral ganglia of Locusta migratoria has a single subunit of 65 K. In contrast Macallan and Lunt (1986) observe four possible subunits in a preparation from the cerebral ganglia of Schistocerca gregaria, and have shown that the irreversible nicotinic ligand

4-(N-maleimide)-[^{3}H]benzyltrimethylammonium binds to a protein of 49 K.

This has lead to some dispute about the similarity of the receptor to that of the vertebrate, Breer et al., (1985) have suggested that the nAChR in the insect is a homooligomer of 65 K subunits, and propose that it may be an evolutionarily primitive form of the nAChR, resembling the ancestral gene from which the four subunits of the vertebrate nAChR, which are all homologous, have evolved (but see Lunt, 1986).

There also seem to be some nAChR which are not αbungarotoxin sensitive, these have been identified electrophysiologically by Goodman and Spitzer (1979; 1980). Similar receptors are known in the vertebrate CNS that bind nicotine and other cholinergic ligands but not αbungarotoxin (for review see Wonnacott, in press).

-Muscarinic AChR-

1.6.3

The presence of a mAChR in the insect CNS has been established by the binding of the radiolabelled ligand tritiated quinuclidinylbenzilate ([^{3}H]QNB). [^{3}H]QNB binding has been shown in the particulate fractions of neural tissues from many insects, including fruitfly heads (Dudai and Ben Barak, 1977; Haim et al.,

1979), housefly heads (Jones and Sumikawa, 1981; Shaker and Eldefrawi, 1981), cockroach ventral nerve cords (Lummis and Sattelle, 1985), the terminal abdominal ganglion from a cricket (Meyer and Edwards, 1980; Meyer and Reddy, 1985) and supraoesophageal ganglia from two different species of locust, (Breer, 1981; Aguilar and Lunt, 1984). The data from which are shown in Table 1.

In all cases binding increased with tissue concentration and was shown to be saturable. Most workers reported a single class of sites of high affinity (i.e. in the nanomolar range). However Aguilar and Lunt (1984) have reported a low affinity binding site (K_D 37.7 nM) in addition to a high affinity binding site (K_D 0.76 nM), which is comparable to those observed by the other workers. It is interesting to note that Meyer and Reddy (1985) who calculated the highest K_D reported for a single class of site mention some evidence of heterogeneous binding and that their figures are close to that that Aguilar and Lunt calculated if they assumed a single class of site.

In all those preparations in which the concentration of nAChR and mAChR have both been determined it has been found that the concentration of nAChR is about an order of magnitude greater than that of mAChR, this situation is the reverse of that found in the vertebrate brain where the mAChR outnumber the binding sites for αBgTx by a similar ratio (Yamamura and Snyder, 1974).

Species	Tissue	K_D(eq.) (nM)	K_D(kin.) (nM)	B_{max} (pmol/mg)	Reference
Drosophila melanogaster	Whole head	2	-	0.08	Dudai and Ben-Barak (1977)
Drosophila melanogaster	Whole head	0.7	0.15	0.065	Haim et al. (1979)
Acheta domesticus	Abdominal ganglion	5.9	-	-	Meyer and Edwards (1980)
Locusta migratoria	Cerebral ganglion	0.77	0.45	0.165	Breer (1981)
Musca domestica	Whole head	2.4	2.76	0.04	Shaker and Eldefrawi (1981)
Schistocerca gregaria	Cerebral ganglion	*0.76 37.7	1.9 9.7	0.114 5.367	Aguilar and Lunt (1984)
Periplaneta americana	Nerve cord	8.0	1.9	0.138	Lummis and Sattelle (1985)
Acheta domesticus	Abdominal ganglion	9.9	4.2	1.9	Meyer and Reddy (1985)

Table 1. QNB binding sites reported in insect neural tissues.
* Aguilar and Lunt (1984) reported two classes of binding sites, the values are given for this two site analysis.

The study of the biochemistry of the mAChR in the insect CNS has, for the most part, been limited to establishing the presence of binding sites for radiolabelled antagonists and a survey of the pharmacology of the binding site, to establish the identity of the binding site as a mAChR. Shaker and Eldefrawi (1981) included a study on the acaricide chlorobenzilate, and some disulphide bond or thiol modifying reagents, which showed no great differences between house fly and verebrate brain mAChRs. Jones and Sumikawa (1981) noted inhibition of [^{3}H]QNB by a wide range of compounds and showed that the responses were mostly rather similar to those in the rat brain except that the [^{3}H]QNB binding in the house fly was rather more stable in some cases.

Lummis *et al*. (1984) have estimated the size of the receptor to be 77.6 K by radiation inactivation target size analysis. This figure is within the range of molecular weights calculated for a wide range of vertebrates (Hoss and Ellis, 1985).

The mAChR in insects has been studied rather less thoroughly than the nAChR, this is partly due to the lower concentrations found, and partly because it has not been possible to identify a muscarinic synapse

by electrophysiology. Indeed for some time the mAChR in insects was a binding site without a function, in a similar way to the mixed pharmacology receptor. Early reports that atropine caused blockade of cholinergic transmission (Callec, 1974) used concentrations (0.1-1.0 mM) that were subsequently shown to be above those required to block radioligand binding to nAChR (K_i 2.0 x 10^{-5} M). More recent studies using improved electrophysiological techniques have shown that lower concentrations of muscarinic antagonists are ineffective at blocking cholinergic transmission in the cockroach ventral nerve cord (Sattelle et al., 1983).

The only electrophysiological evidence for a functional role for the mAChR is at the neuromuscular junction on the extensor tibiae muscle of the metathoracic leg of Schistocerca gregaria (Fulton and Usherwood, 1977; Fulton, 1982). ACh was found to have two effects, an initial surge in the frequency of miniature excitatory post-synaptic potentials, which was of short duration, followed by a slower increase in frequency lasting several minutes. Fulton (1982) found that the initial surge in frequency was induced by nicotine and blocked by tubocurarine and thus assigned it a nicotinic response, however the slower more prolonged increase in frequency was mimicked by the application of acetyl-ß-methyl choline, a specific muscarinic agonist, but not by nicotine, nor was the effect blocked by tubocurarine. These effects were

calcium dependent and Fulton (1982) considered them to be due to receptors presynaptic to the neuromuscular junction.

Another functional role for the mAChR in the insect CNS has been described by Breer and Knipper (1984). Breer and Jeserich (1980) had previously described the preparation of insect synaptosomes (pinched off nerve terminals), and their uptake of [^{3}H]-choline has been described (Breer, 1982). Breer and Knipper (1984) studied the release of acetylcholine from a synaptosomal preparation. It was found that muscarinic drugs modulated the release of acetylcholine that was stimulated by 35 mM K^+. The antagonist atropine increased the release, whereas the agonist oxotremorine inhibited it. These results were interpreted to suggest the presence of a presynaptic autoreceptor at the nerve terminal with a muscarinic pharmacology, similar to that described in mammalian brain (Nordström and Bartfai, 1980).

-Second Messengers in the Insect CNS-

1.7

Both adenylate cyclase and PI turnover have been demonstrated in insects. However neither had been shown to be coupled to mAChRs at the time this programme commenced.

-Phosphoinositide metabolism-

1.7.1

Some of the important experiments in deducing the role of PI metabolism were carried out in insect tissues (Fain and Berridge, 1979; Berridge, 1983; Berridge *et al.*, 1984). The tissue used was the salivary gland of the blowfly, and the response was evoked by 5-hydroxytryptamine. Some attempts have been made to find a neurotransmitter-regulated PI metabolism in the insect CNS. Killian and Schacht (1979) found polyphosphoinositides in sensory structures of the moth *Agrotis ypsilon*. Yoskioka *et al.* (1985), however, found that phosphatidylinositol phosphodiesterase, the enzyme required for the PI response, was localized almost entirely to the eyes in *Drosophila melanogaster*. Both manual dissection and genetic dissection, using the mutant *sine oculus*, showed that more than 90% of the enzyme activity is in the eye.

In the metathoracic ganglion of Schistocerca gregaria Trimmer and Berridge (1985) showed that [^{3}H]inositol was incorporated into the inositol phosphates associated with second messenger responses. However they were unable to show any effect on the concentrations of these compounds by a range of neurotransmitters. The muscarinic antagonist atropine reduced inositol phosphate production and this was taken as some evidence for muscarinic regulation. To prove muscarinic control requires demonstration of agonist enhanced turnover, especially as Imhoff and Rossignol (1983) have shown that atropine at concentrations lower than that used by Trimmer and Berridge (1985) (10μM as opposed to 120μM) inhibits the PI turnover stimulated by α-adrenergic agonists.

Ross and Brady (1986) followed the incorporation of ^{32}P into the phospholipids of the nerve cord of the cricket Acheta domesticus. They found that under the influence of both acetylcholine and the anticholinesterase insecticide dichlorvos the activity of most phospholipids was increased, but not significantly. The interpretation of their results is difficult because they measured only the total activity in each class of phospholipid rather than the specific activity so that it cannot be known whether the increases are due to increased specific activity or absolute amount of phospholipid.

Thus although there is some evidence for a

muscarinically linked PI metabolism its existence has not been proved.

-Adenylate cyclase-

1.7.2

A neuronal adenylate cyclase has been shown in many insect species. It was first shown by Nathanson and Greengard (1983) in the cockroach, and has been confirmed by many workers since (for review see Smith and Combest, 1985) these workers have concentrated on the stimulation of adenylate cyclase by octopamine. This has been shown in Schistocerca gregaria cerebral ganglia by Morton (1984), who found that several putative monoamine neurotransmitters stimulated adenylate cyclase activity.

Suter (1986) has tested a range of neurotransmitters for effects on the adenylate cyclase from the thoracic ganglion of Schistocerca gregaria, he found that acetylcholine had no significant effect on adenylate cyclase activity. The incubation conditions used did not include any sodium, and whereas stimulation of adenylate cyclase activity is not sodium dependent the muscarinic downregulation is (Jakobs et al., 1979). Thus it is not known whether insect tissues possess a muscarinically regulated adenylate cyclase.

-What next?-

1.8

It was against this background of a comparative lack of information concerning the neurochemistry of the insects that this study was commenced. The bulk of this work is concerned with the muscarinic acetylcholine receptor of the desert locust Schistocerca gregaria, the second messengers to which it is coupled and its interaction with the membrane.

CHAPTER 2.

GENERAL METHODS

-Tissue Preparation-

2.0

All experiments were carried out using the supraoesophageal ganglion from the adult desert locust Schistocerca gregaria, supplied by Larujon ltd., Colwyn Bay.

Locusts were anaesthetised with carbon dioxide before being decapitated. The anterior portion of the cuticle, from above the labium through the eyes was shaved off with a scalpel to expose the supraoesophageal ganglion. The ganglion was freed from its connections to the eyes and the ventral nerve cord, and lifted out with Dumont watch-makers forceps.

One hundred ganglia have a wet weight of approximately 250mg.

-Preparation of a P_2 membrane fraction from locust-supraoesophageal ganglia-

2.1

This procedure is based on that of Filbin et al. (1984). Ganglia were homogenised in 10 ml of buffer (50 mM sodium phosphate, pH 7.2, with 2 mM EDTA), for every 100 ganglia, in a motor driven Potter-Elvejhem homogeniser with a 'Teflon' pestle and a radial clearance of 0.15 mm. Forty strokes were made at

500 rpm.

A low speed centrifugation (500 x g, 10 min., 4°C) produced a nuclear pellet (P_1) and a supernatant (S_1). The pellet was resuspended in 5 ml of buffer and recentrifuged (500 x g, 10 min., 4°C). The combined supernatants were strained through nylon bolting cloth (159 µm mesh) before a high speed centrifugation (100,000 x g, 40min., 4C) this produced a membrane pellet (P_2) and a supernatant (S_2).

-[^{3}H]-Quinuclidinylbenzilate binding assay-

2.2

d-l-Quinuclidinyl[phenyl-4-^{3}H]benzilate ([^{3}H]QNB) was obtained from Amersham International at an activity of 30-60 Ci.mmol^{-1}.

The P_2 membrane pellet was resuspended in buffer with a hand-held homogeniser so that the tissue from 100 locusts was homogeneously distributed in 20 ml of buffer.

Unless otherwise specified portions (500µl) were incubated at 25°C in the presence of 2nM [^{3}H]QNB to assess total binding. In order to estimate that portion of the binding that does not result from interactions with the muscarinic acetylcholine receptor binding was measured in the presence of 0.1mM atropine.

It had previously been shown that the binding

of [^{3}H]QNB reaches equilibrium after 1 h. (Aguilar & Lunt, 1984). After this time the [^{3}H]QNB bound to the membrane was separated from that in solution by filtration through glass fibre filters (Whatman GFB) under reduced pressure (Millipore 12 filter manifold). Any further free [^{3}H]QNB was washed through with two washes of 5 ml ice-cold buffer.

The filters were then oven-dried (110^{o}C, 15 min) and placed in plastic scintillation vials with 5 ml of scintillant (0.5% PPO in toluene, w/v). The activity of the samples was determined by liquid scintillation counting in a LKB 1217 Rackbeta liqid scintillation counter. The counting efficiency for [^{3}H]QNB was 37% (using the preset ^{3}H window) and quenching was found to be constant by external standards ratio, enabling the bound label to be determined from comparison with a known ammount of [^{3}H]QNB counted in parallel.

CHAPTER 3.

MEMBRANE LIPID ANALYSIS

-Extraction of the lipids from locust P_2-

3.1

The P_2 from 100-150 locust ganglia was prepared as described, but not resuspended in buffer. Instead it was scraped from the ultracentrifuge tubes, any remaining being washed out in the minimum possible distilled water, to avoid the presence of inorganic phosphate and EDTA, which might have interfered with the lipid extraction or phosphate assay.

The following method is based on the lipid extraction developed by Folch et al. (1957) using the modifications described by Veerkamp and Broekhuyse (1976). All solvents were redistilled before use and all stages were performed on ice to reduce oxidation of the lipids.

The sample was homogenised in ten volumes of $CHCl_3$:CH_3OH (1:1, by volume) then an equal volume of $CHCl_3$:CH_3OH (3:1, by volume) was added and the mixture homogenised again. Precipitated protein was removed by centrifugation (500 xg, 5 min.). The supernatant was retained and the pellet was resuspended in 3 ml of $CHCl_3$:CH_3OH (2:1, by volume) and recentrifuged (500 xg, 5min)

The combined supernatants were shaken with 0.2 volumes of 0.1 M $MgCl_2$(aq.). The phases were separated by centrifugation and the upper phase removed by aspiration. The interface was washed with 2 x 2 ml

'Folch upper phase' ($CHCl_3$: CH_3OH: 0.1M.$MgCl_2$(aq.); 3:48:47; by volume). The lower phase was then concentrated on a rotary evaporator, fitted with a nitrogen bleed for repressurising to avoid oxidation of the fatty acids. To the small volume remaining benzene:CH_3OH (4:1, by volume) was added until a homogenous mixture was obtained. This allowed removal of the residual water by codistillation on the rotary evaporator.

The lipids were then transferred to a sample bottle in 3 x 1.5 ml washes of $CHCl_3$:CH_3OH (2:1). This was then evaporated under a stream of nitrogen and the lipids redissolved in 2 ml benzene:CH_3OH (4:1, by volume) before being stored, under nitrogen at -80°C.

-Phospholipid separation-

3.2

The neutral lipids were removed from the total lipid extract obtained from the Folch extraction by washing the dry lipid film with acetone, neutral lipids are soluble in acetone whereas phospholipids are not (Lovelock et al., 1960).

The different classes of phospholipid were separated by the method of Skipski et al. (1964). This involved single dimension thin layer chromatography on plates (20 cm x 20 cm) coated with silica gel H to a depth of 0.5 mm. The developing solvent was $CHCl_3$: CH_3OH: CH_3CO_2H: H_2O (25:15:4:2, by volume) and was allowed to equilibrate with its vapour in the glass tank for at least 1 h before the plate was developed. The plates were activated in an oven at 100°C for at least 1 h before use. The sample, dissolved in two drops of $CHCl_3$: CH_3OH (2:1, by volume), was immediately applied in a 7 cm horizontal band. This has to be done rapidly because retaining maximum activation of the plate is essential for a good separation. The development took 2 h.

The phospholipids were visualised by exposure to iodine vapour. The bands were then scraped onto glazed paper and the silica transferred to glass centrifuge tubes containing 3 ml of the developing solvent. Parallel areas of silica from the plate were

used as blanks. The lipids were extracted by mixing the silica with the solvents given below and then centrifuging and collecting the solvent. The solvents employed were:

1. 3 ml of developing solvent.
2. 2 ml of developing solvent.
3. 2 ml of CH_3OH.
4. 2 ml of CH_3OH: CH_3CO_2H: H_2O (94:1:5, by volume).

The combined solvents were concentrated on a rotary evaporator, fitted with a nitrogen bleed, and the residual water removed by addition of benzene:CH_3OH (4:1, by volume) and further evaporation as described previously. The samples were stored at -80°C under nitrogen.

-Quantitative analysis of phospholipids-

3.3

The samples were evaporated to dryness over a boiling water-bath in boiling tubes with an anti-bumping granule. Four drops of concentrated sulphuric acid were added followed by two drops of 72% perchloric acid. The tubes were then heated on a 'Kjeldahl ashing rack' until the mixture was seen to be clear, colourless and refluxing on the sides of the tubes.

The samples were allowed to cool and then made up to a volume of 5 ml in a volumetric flask. Samples (2 ml) were taken in duplicate, to analyse the inorganic phosphate content.

To the 2 ml samples was added a 2 ml portion of a reagent, of composition:

10 ml 3 M sulphuric acid

20 ml water

10 ml 2.5% ammonium molybdate

10 ml 10% ascorbic acid

The tubes were mixed and incubated in a shaking waterbath for 90 min. at 37°C. The absorbance at 820 nm was then read and the phosphate concentration calculated by comparison with a standard curve prepared from inorganic phosphate.

-Determination of the fatty acid composition- -of membrane phospholipids-

3.4

-Hydrolysis of the phospholipids-

3.4.1

Hydrolysis to produce free fatty acids was performed by the method of Lovelock et al. (1959). The samples of phospholipids were dissolved in a mixture of aqueous ethanol (50%, by volume; 5 ml) and potassium hydroxide (40%; w/v; 1 ml) and the mixture heated at 60°C for 4 h After the mixture had cooled to room temperature 3 ml of water and 2 ml of 5 M sulphuric acid were added and the solution thoroughly mixed. The free fatty acids were then extracted from the mixture with three 5 ml portions of 40-60 petroleum ether.

The pooled petroleum extracts were then concentrated on a rotary evaporator and dried as before with addition of benzene:methanol (4:1, by volume).

-Methylation of free fatty acids-

3.4.2

The samples of free fatty acids were dissolved in 5 ml of diethyl ether. A solution of diazomethane in diethyl ether (prepared from N-methyl-N-nitrosotoluene-4-sulphonamide by the method of Vogel, 1957) was added dropwise until the yellow colour persisted, and the

mixture was allowed to stand for 20 min at room temperature. The excess diazomethane and the ether were removed by distillation on a rotary evaporator fitted with a nitrogen bleed. The methyl esters were stored in benzene:methanol (4:1, by volume) at -80°C.

-Gas-liquid chromatography of- -fatty acid methyl esters-

3.4.3

Gas-liquid chromatography (glc) of the methyl esters is the preferred method of analysing fatty acids. It has a very high sensitivity, enabling less than 1 µg of an individual methyl ester to be detected easily, while also separating a complex mixture of compounds. This allows a quantitative determination to be made of the composition of fatty acids in a sample.

Apparatus: The methyl esters were analyzed using a Pye Series 104 gas chromatograph, fitted with a flame ionization detector. The samples were chromatographed on either:

1. 15% (w/w) EGSS-X on Chromosorb W (100-120 mesh) packed glass column (7' x 3 mm i.d.). Temperature 178°C. Carrier gas nitrogen 40 ml/min.

2. 5% (w/w) Apiezon L on Chromosorb W (100-120 mesh) packed glass column (5' x 3 mm i.d.). Temperature 200°C. Carrier gas nitrogen 40 ml/min.

Samples: The samples were dissolved in n-hexane and a known ammount of methyl-heptadecanoate was added as an internal standard . The samples were then concentrated under a stream of nitrogen to a volume of a few microlitres. A small sample (<0.5 µl) was then loaded onto the column using a microlitre syringe.

Quantification: The mass of each methyl ester present was found to be proportional to the area under the peak on the recorder trace. The area was estimated by triangulation and the absolute quantity calculated by proportion to the peak of the internal standard. The mass of the internal standard added was of the same order of magnitude as the amounts of individual fatty acids present in the sample.

-Analysis of the neutral lipids of locust ganglia-

3.5

The acetone soluble lipid fraction (see section 3.2) was dried and stored in benzene:methanol (4:1, by volume) at -80°C.

3.5.1

The different classes of neutral lipid were separated by thin layer chromatography by the method of Broekhuyse (1972) (glass plates 20 cm x 20 cm, 0.5 mm silica gel G 'nach Stahl', developed with petroleum ether (b.p. 40-60°C): diethylether: formic acid; 80:20:2, by volume).

The band corresponding to the sterols was scraped off and the sterols recovered by elution with 3 x 5ml of developing solvent.

3.5.2

The sterols were analysed by gas-liquid chromatography of their trimethylsilyl derivatives. These derivatives were prepared with bis(trimethylsilyl)trifluoroacetamide (BSTFA, supplied by Sigma Chemical Co. Ltd.). The BSTFA is supplied as a solution in pyridine and 0.5 ml of the solution was used to dissolve the dry sterol component from one preparation in a glass reactivial, this was then sealed and incubated for 30 min at 60°C in a water bath.

Apparatus: The gas-liquid chromatography was performed on a Pye Unicam PU4500 series machine using the following conditions.

Column: 25m capillary column.

Active phase: S.E.30

Carrier gas: Helium.

Flow rate: 1 ml min^{-1}.

Temperature: 230°C for 5min then increasing at 4°C min^{-1} to 300°C.

Detection: Flame ionization detector at 350°C.

Quantification: The mass of each sterol derivative was known to be proportional to the area under the peak on the recorder trace. This area was given by the integrator fitted to the Pye Unicam PU4500, and the absolute value calculated by proportion to the peak of the internal standard (ergosterol). The mass of the internal standard was of the same order of magnitude as that of sample steroids.

-Membrane phospholipid composition-

3.6

The thin-layer chromatogram obtained by the method of Skipski et al. (1964) showed bands corresponding to the major classes of phospholipid found in mammals (see Fig. 7). Initial experiments showed no loss of phospholipid with the acetone wash but a noticeable reduction in the intensity of staining by iodine at the solvent front consistent with the loss of neutral lipids. Also extracted in the acetone wash was a yellow component which ran at the solvent front and may be responsible for the coloration of the ganglion.

The quantity of inorganic phosphate which remained after the ashing of each phospholipid fraction was taken to be proportional to the number of moles of phospholipid in each phospholipid class. This calculation is based on the assumption that each mole of phospholipid contains one mole of phosphorus atoms.

The results of the phosphate determination are shown in Table 2.

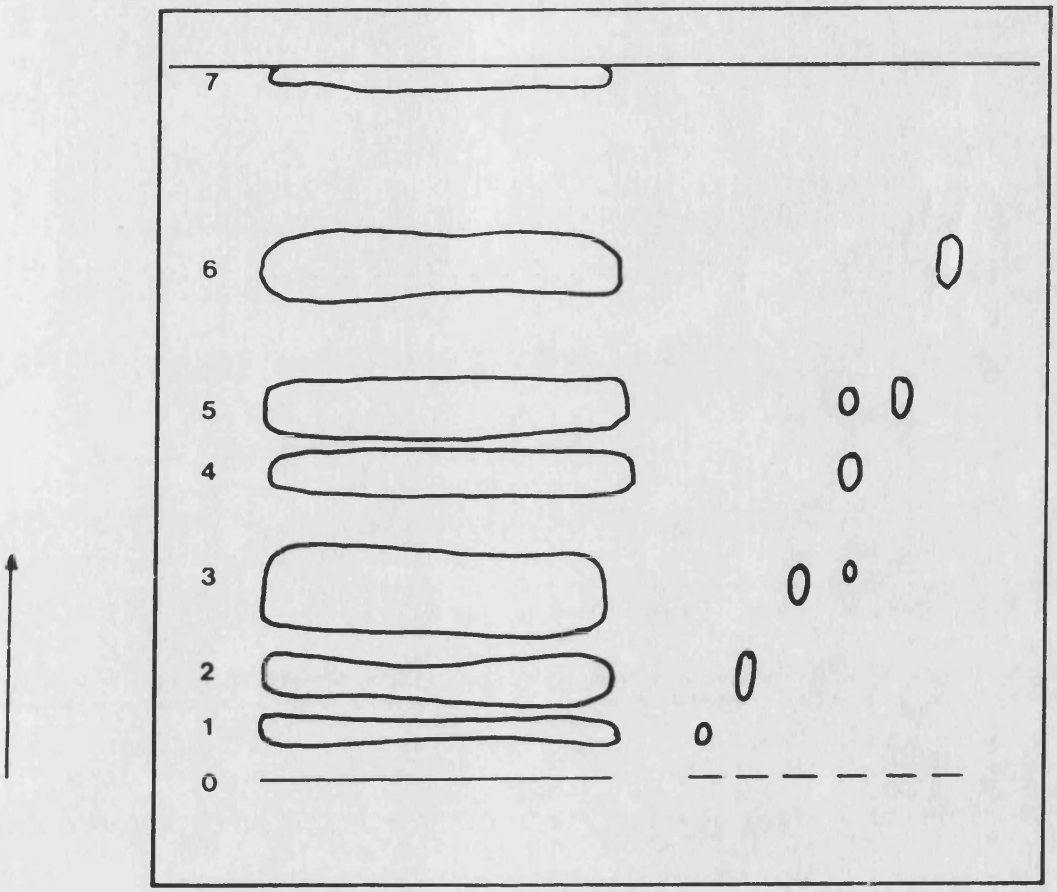

Figure 7. Locust membrane phospholipids.
Thin-layer chromatography by the method of Skipski *et al*. (1964).

0. Origin. 1. Lysophosphatidylcholine. 2. Sphingomyelin. 3. Phosphatidylcholine. 4. Phosphatidylserine. 5. Phosphatidylinositol. 6. Phosphatidylethanolamine. 7. Solvent front with neutral lipids and cardiolipin.

Phospholipid	% Composition by Inorganic Phosphate.
Lysophosphatidylcholine	8.7 ± 3.3
Sphingomyelin	7.6 ± 3.0
Phosphatidylcholine	31.1 ± 8.7
Phosphatidylinositol	6.3 ± 2.3
Phosphatidylserine	14.4 ± 4.6
Phosphatidylethanolamine	29.3 ± 9.1
Cardiolipin	2.5 ± 1.5

Table 2. Composition of the Phospholipids from locust ganglionic membranes.

The results are the means of 4 separate experiments done in duplicate. The errors are the standard deviations from the means.

-Composition of fatty acids from membrane phospholipids-

3.7

The fatty acid derivatives were identified by comparing the retention times against standards on both the polar and non-polar columns (see section 3.4.3). There was some interference with the determination of the short chain fatty acids from tailing of the solvent peak, probably due to the presence of low molecular weight contaminants. This only seriously affected the determination of palmitic acid ($C_{16:0}$) a minor component in the fatty acid composition and values were obtained where palmitic acid was present in amounts greater than 5 %.

No fatty acids greater than 20 carbon atoms in length were found in these preparations. An experiment using phosphatidylethanolamine from rat brain membranes showed that docosahexenoic acid ($C_{22:6}$) was detectable using the method described. Confirming that the lack of these long chain polyunsaturated fatty acids in the locust samples, was not due to faulty technique.

The compositions determined are shown in Table 3.

Phospho-lipid	Fatty Acid Composition							
	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:4
LPC	15	10	2	10	51	2	0	7
SM	Tr	Tr	13	49	29	8	0	0
PC	21	0	0	25	28	11	16	Tr
PI	14	0	0	8	63	6	Tr	Tr
PS	Tr	Tr	5	28	12	4	17	34
PE	11	1	0	14	23	12	36	0
CL	34	3	Tr	20	22	7	14	0

Table 3. Fatty Acid Composition of the classes of phospholipid found in the P2 fraction.

The values are the means of results from three different preparations and are expressed as % of the total fatty acid recovered for that class of phospholipid. The variations between preparations were within 20% of the value shown.

Tr. indicates that the phospholipid was present in small quantities but not quantifiable, or less than 1% of the fatty acid.

-The neutral lipid composition of the- -ganglionic P_2 membrane fraction-

3.8

The thin-layer chromatograms of the acetone soluble lipid fraction (see section 3.5.1) showed bands corresponding to several different neutral lipid classes but no lipid remaining at the origin where any phospholipid would be expected to stay (see Fig. 8) A preliminary experiment in which the total lipid extract was applied showed phospholipid but no bands of neutral lipid additional to those observed in the acetone extract.

The bands observed corresponded to (by comparison with standards):

1. Solvent front with yellow lipid.
2. Triglycerides.
3. Free fatty acids.
4. Sterols.
5. Monoglycerides or Diglycerides.

The band assigned as monoglycerides or diglycerides was very faint. There was no indication of sterol esters, which would have been expected to run between the triglyceride band and the solvent front (Broekhuyse, 1972). The presence of some remaining yellow lipid and some glycerides not usually associated with membranes suggests that the P_2 fraction may contain

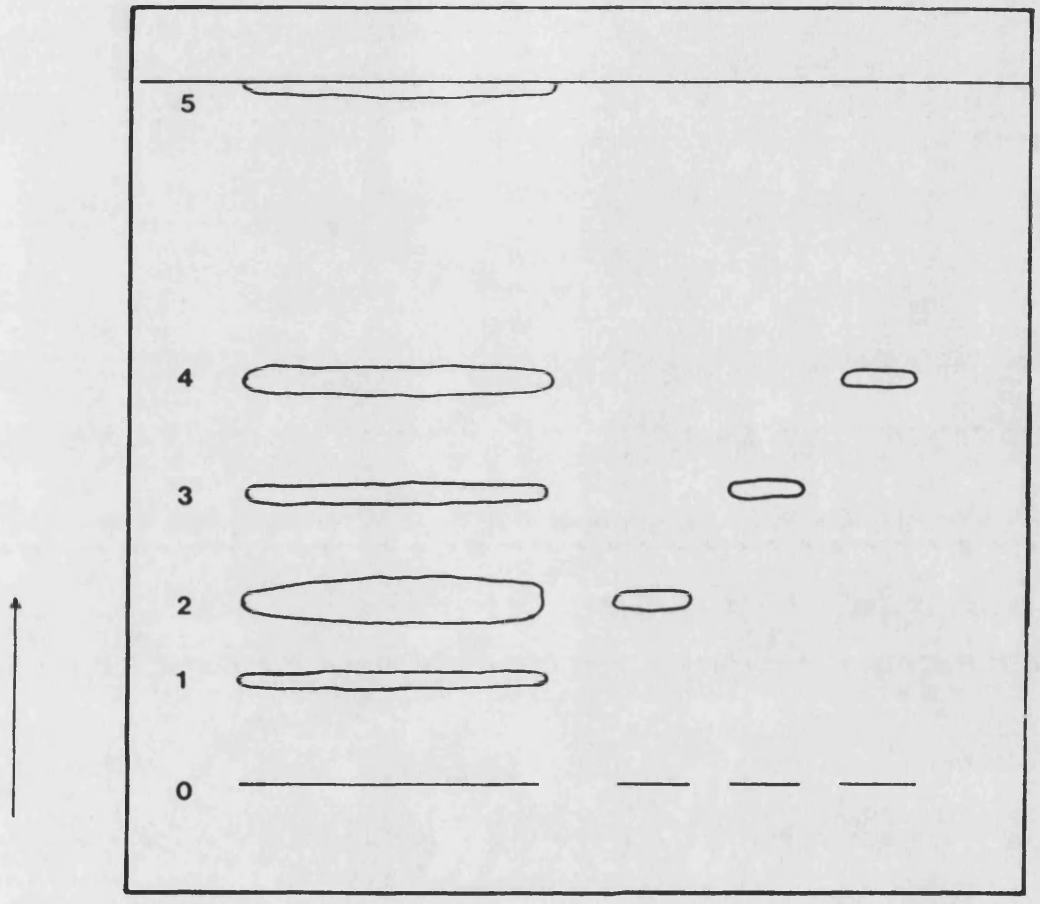

Figure 8. Neutral lipids from the P_2 fraction. Thin-layer chromatography by the method of Broekhuyse (1972).

0. Origin. 1. Monoglycerides or diglycerides. 2. Cholesterol. 3. Free fatty acids. 4. Triglycerides. 5. Solvent front.

fragments of the fatty tissue which surrounds the ganglion.

-Analysis of the sterol component-

3.9

Gas-liquid chromatography of the derivatives of the sterol fractions from the thin-layer chromatograms showed 3 major components, the largest of which had the same retention time as cholesterol. The other two components were not identifiable by comparison to the available standard sterols. The composition is shown in Table 4.

Sterol Component	Retention Time (sec)	% Composition (by peak area)
Cholesterol	1500	64 ± 12
Unknown 1	1543	25 ± 7
Unknown 2	1601	9 ± 4

Table 4. The composition of sterols from locust ganglionic membranes. Results are the mean of three seperate experiments and the errors are the standard deviations from the mean.

-The lipid composition of the membranes-

3.10

Compared with the membrane components of mammalian systems those of insects have been little studied. Published data for insect lipid compositions generally refer to the whole insect (for example see Fast, 1964; 1970 and Bridges, 1972).

In Schistocerca gregaria the only membrane phospholipid analysis previously available was for the mitochondria from flight muscles (Kallapur et al., 1982). Kallapur et al. (1982) analysed the lipid composition of mitochondria from locusts raised at two different temperatures, and found that the composition varied in the relative proportions of PC and PE.

The only report of membrane lipid analysis from neural tissue of a locust is that of Breer and Jeserich (1984), from a synaptosomal preparation from Locusta migratoria. They only measured the composition of the major phospholipid classes, and did not study the fatty acid composition. Within the errors quoted in both this study and that of Breer and Jeserich (1984) the phospholipid composition of both preparations is the same.

Although the P_2 membrane fraction used in this study is expected to contain many mitochondria the phospholipid composition resembles that determined for the synaptosomal preparation of Breer and Jeserich

(1984) more closely than that of the mitochondria from Schistocerca gregaria (Kallapur et al., 1982). Both the preparation under study in this work and that of Breer and Jeserich (1984) do, however, contain small proportions of cardiolipin, a phospholipid usually thought to be a mitochondrial marker.

Both these lipid compositions are similar to the values found in neuronal membranes from vertebrates (for example see Breckenridge et al., 1972). Thus locust neural membranes appear to have similar classes of phospholipids in similar proportions to those found in mammalian neuronal membranes. The main difference between this preparation and that of the rat brain preparation of Breckenridge et al. (1972) is the higher proportion of lysophosphatidylcholine (8.7% in the locust compared with 1.0% in the rat) this may be due to phospholipase activity in the ganglia before extraction of the lipids, and may also be the source of the free fatty acids found in the preparation.

The fatty acid composition of the different classes of phospholipid do show some significant differences when compared with the values for the rat (Breckenridge et al., 1972). In the rat docosahexenoic acid ($C_{22:6}$) is a major constituent of the phospholipid fatty acids, forming 32.4% of the fatty acid from PE and 34.1% of the fatty acid from PS. In the locust preparation, however, $C_{22:6}$ was never detected. By contrast Breckenridge et

al. (1972) did not report the detection of linolenic acid ($C_{18:3}$) which in the locust preparation accounts for 36% of the fatty acid from PE and significant proportions of the fatty acids from both PC and PS.

Indeed no fatty acids could be detected in the locust preparation which were greater than twenty carbon atoms in length, and at that length only arachidonic acid ($C_{20:4}$) was detected. and composed 5% of the total fatty acid.

Arachidonic acid metabolism has been the subject of many reports in the literature for insects because of its importance as a precursor for prostaglandin production (for example see Wakayama et al., 1985). In the phospholipid fraction the presence of $C_{20:4}$ in PI is especially of interest as it has been suggested that in receptor-linked phosphoinositide turnover the liberated diacylglycerol may not only stimulate protein kinase C activity but may also be hydrolysed to release $C_{20:4}$ leading to an eventual increase in prostaglandin levels (for review see Berridge, 1984).

In this preparation $C_{20:4}$ was observed in the PI fraction but only in very small amounts, averaging less than 1% of the fatty acids from PI. Wakayama et al. (1985) working on the housefly found that [^{3}H]arachidonic acid was rapidly incorporated into the phospholipids of houseflies but showed no preference for the PI/PS spot on their thin-layer chromatograms, the

developing system used did not separate these phospholipids. In this study, however, more than 90% of the $C_{20:4}$ found to be present in the PS. This may reflect differences either between the metabolisms of the two species, Musca domestica and Schistocerca gregaria, or between the different tissues as Wakayama et al. (1985) used whole insects in their study whereas this work is on ganglionic membranes specifically.

It is possible that these results underestimate the proportion of $C_{20:4}$ in the PI fraction. Abdel-Latif et al. (1974) have shown that in rat brain the phosphoinositides containing $C_{20:4}$ are apparently subject to a much greater rate of turnover than those without. If this is the case in the locust cerebral ganglion then the high turnover of phosphoinositides which may have occurred after dissection of the ganglia might have resulted in a specific depletion of the PI which contained $C_{20:4}$.

The specificity of the turnover of PI for that part of the fraction which contain $C_{20:4}$ might be tested. Labelling the phospholipids in vivo by feeding or injecting live locusts with either ^{32}P or [^{3}H]inositol would allow the incorporation of radiolabel without the trauma of dissection. Rapid freezing, and dissection of the still frozen cerebral ganglion followed immediately by extraction of the phospholipids should preserve the initial PI composition. It would then be possible to separate the PI by tlc and to

further fractionate the PI by tlc on a silver nitrate treated support, as reported by van Rooijen et al. (1985).

As the experiments involving the incorporation of ^{32}P into the phospholipid pool of the locust cerebral ganglion have shown, the turnover rate of most of the phospholipids is much slower than that of PI. This indicates that their major role is probably a structural one. The composition of fatty acids which make up the hydrophobic core of the lipid bilayer in the biological membrane is commonly held to be responsible for its physical properties (Emmelot and van Hoeven, 1974; Helmreich and Elson, 1984).

There is no method of predicting the physical properties of a membrane from the lipid composition alone, because the complex mixture of components, phospholipid, sterol and protein all contribute to the fluidity and stability of the membrane. It is generally the case, however, that shorter and more unsaturated fatty acids produce a more fluid membrane.

Comparing the fatty acid compositions of locust ganglionic membranes (this study) with those of rat brain synaptic membranes (Breckenridge et al., 1972) the number of double bonds in the fatty acids from the locust preparation is fewer but the carbon chains are shorter.

Cholesterol has an important influence on

membrane properties (Emmelot and van Hoeven, 1974; Harris and Schroeder, 1981) decreasing the membrane fluidity but preventing the phase change to a rigid gel. It is also believed to be particularly important in the activity of some receptors, Zabrecky and Raftery (1985) have shown that depleting the cholesterol from membranes decreased nAChR function. The rat brain synaptosomal membranes have a cholesterol:phospholipid ratio of 0.22 by mass (Breckenridge et al., 1972).

In the locust preparation used here the sterol fraction was composed of three components. The major species was cholesterol, which comprised 64% of the total. Thus in this study the ratio of sterol:phospholipid was approximately 0.6 for all sterol or 0.4 for cholesterol alone.

This study of the composition of the lipid fraction of the neural membranes from the locust cerebral ganglion shows that there are differences in the composition from that of vertebrate systems which may be sufficient to give some difference in the properties of the plasma membranes in which neurotransmitter receptors are located. This may reflect the difference in physiology of the two species, locusts are poikilotherms and not only do all their metabolic processes have to be able to function at temperature which is, in this case, 12°C below that of the rat (the locusts were kept at 25°C) but also have to be able to adjust to changes in temperature.

-Further work-

3.12

This preliminary work on the lipid composition of the membranes from insect tissues has established that there is a difference from the composition of membranes from mammals. However there are classes of lipid which have not been fully investigated. The glycolipids have not been studied at all, apart from a statement in the work of Breer and Jeserich (1984) that they could not detect any gangliosides. Similarly the other types of linkage of hydrocarbon chains to phospholipids, the ether linked types have been identified in whole insects (for review see Downer, 1985) but were not investigated in this study. Methods for the analysis of these minor classes of phospholipid do exist but may need to be modified to compensate for the very small amounts of tissue available in insect neurochemistry.

Some work has been done on the variation in the lipid composition of insects bred at different temperatures (Downer, 1985), however this work has mostly been performed using whole insects and the nervous system which relies so heavily on membrane situated events might show more dramatic changes.

CHAPTER 4.

MEMBRANE PROPERTIES

-Measurement of membrane anisotropy-
-by fluorescence polarization-

4.1

-Tissue sample preparation-

4.1.1

A P_2 membrane pellet from 50 cerebral ganglia was resuspended in 1.1 ml of 0.25 M sucrose (4°C) and layered on top of 4.5 ml of 1.2 M (4°C) sucrose in a centrifuge tube for the SW.50.1 rotor for a Beckman L5-50B Ultracentrifuge. The sample was centrifuged at 100,000 x g for 60 min (4°C).

Three fractions were collected from the tube after centrifugation:

1. From the surface of the 0.25 M sucrose.
2. From the interface between the 0.25 M and 1.2 M sucrose.
3. The pellet produced at the bottom of the tube.

These fractions were each diluted in phosphate buffer and centrifuged (30 min, 100,000 x g, 4°C). The resulting pellets were resuspended in phosphate buffer and samples assayed for protein concentration and $[^3H]$QNB binding.

Fraction 2 was resuspended to a protein concentration of 100µg protein ml^{-1}. To 1 ml of this was

added 1 µl of 1,6-diphenylhexatriene solution (DPH, 2 mM in tetrahydrofuran). The sample was then incubated for 30 min in the dark at room temperature.

-Anisotropy determination-

4.1.2

Fluorescence polarization measurements were taken on samples in the presence and absence of the fluorescent probe DPH to allow for light scattering by the turbid sample.

A sample of fraction 2 (300 µl) was added to 700 µl of sodium phosphate buffer in the cuvette for the fluorescence polarization measurement.

The fluorescence polarization was measured in a standard Aminco-Bowman spectrophotofluorometer, which had been fitted with a pair of polaroid filters which could be turned through 90^{o} to change the plane of polarization of either the irradiating light or the detected fluorescence (see Fig. 9).

The sample was irradiated with monochromatic light (335 nm) and fluorescence intensity measured (430 nm). The temperature of the sample was varied by means of a water jacket around the cuvette connected to a circulating water bath. The temperature of the sample was measured by a thermocouple placed into the sample, care was taken to ensure that the thermocouple was outside the path of the irradiating light to prevent

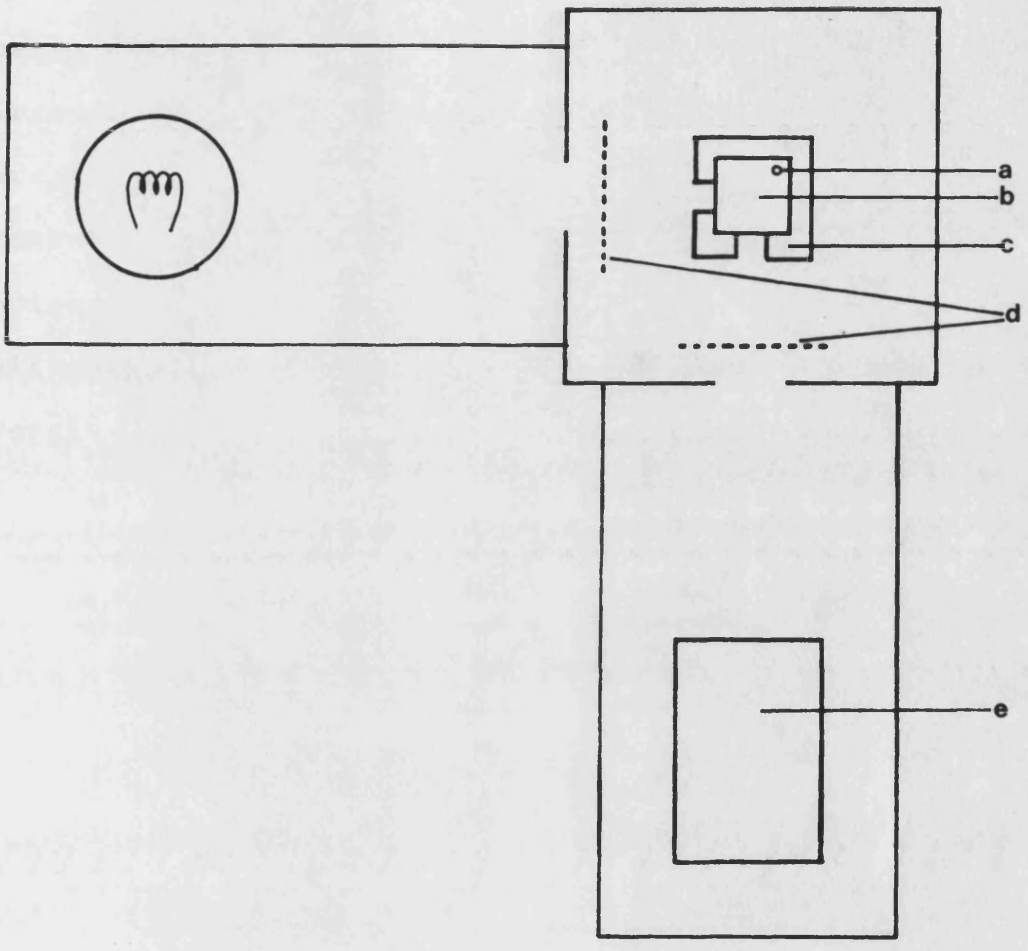

Figure 9. Diagrammatic representation of the apparatus used for the measurement of fluorescence polarization.

a. Thermocouple; b. Sample; c. Water-jacket; d. Polaroid filters; e. detector.

interference.

At each temperature point readings of the fluorescence intensity were taken at all four permutations of the planes of polarization of irradiation and fluorescence. That is with both irradiation and fluorescence vertically polarized, with both horizontally polarized, with irradiation vertically polarized and fluorescence horizontally polarized and with irradiation horizontally polarized and fluorescence vertically polarized.

-The effect of diacetal on [^{3}H]-QNB binding-

4.2

The P_2 fraction was prepared in borate buffer (50 mM boric acid plus 50 mM sodium tetraborate to pH 7.8) and incubated with varying concentrations of diacetal (1.0-0.01 mM) and the time course of any change in [^{3}H]QNB binding was followed, the experiments were based on the procedure of Millan *et al*. (1983). The filter assay employed was that given above (section 2.2).

The percentage loss of [^{3}H]QNB binding was expressed relative to samples incubated in the absence of diacetal. In experiments using low concentrations of diacetal no significant effect on [^{3}H]QNB binding was observed. However at a concentration of 50 mM the time course showed an initial rise in binding before falling gradually (see Figure 10).

Some samples were incubated with both diacetal and atropine (0.1 mM), demonstrating that the effect of diacetal was not due to any change in non-specific binding.

In order to check whether the effect produced by diacetal was due to a 'solvent effect' parallel experiments were run substituting acetone for diacetal. The time course of the effect produced by 50 mM acetone is shown in Figure 10. The shape of the curve is similar to that produced by diacetal, but the deviations from

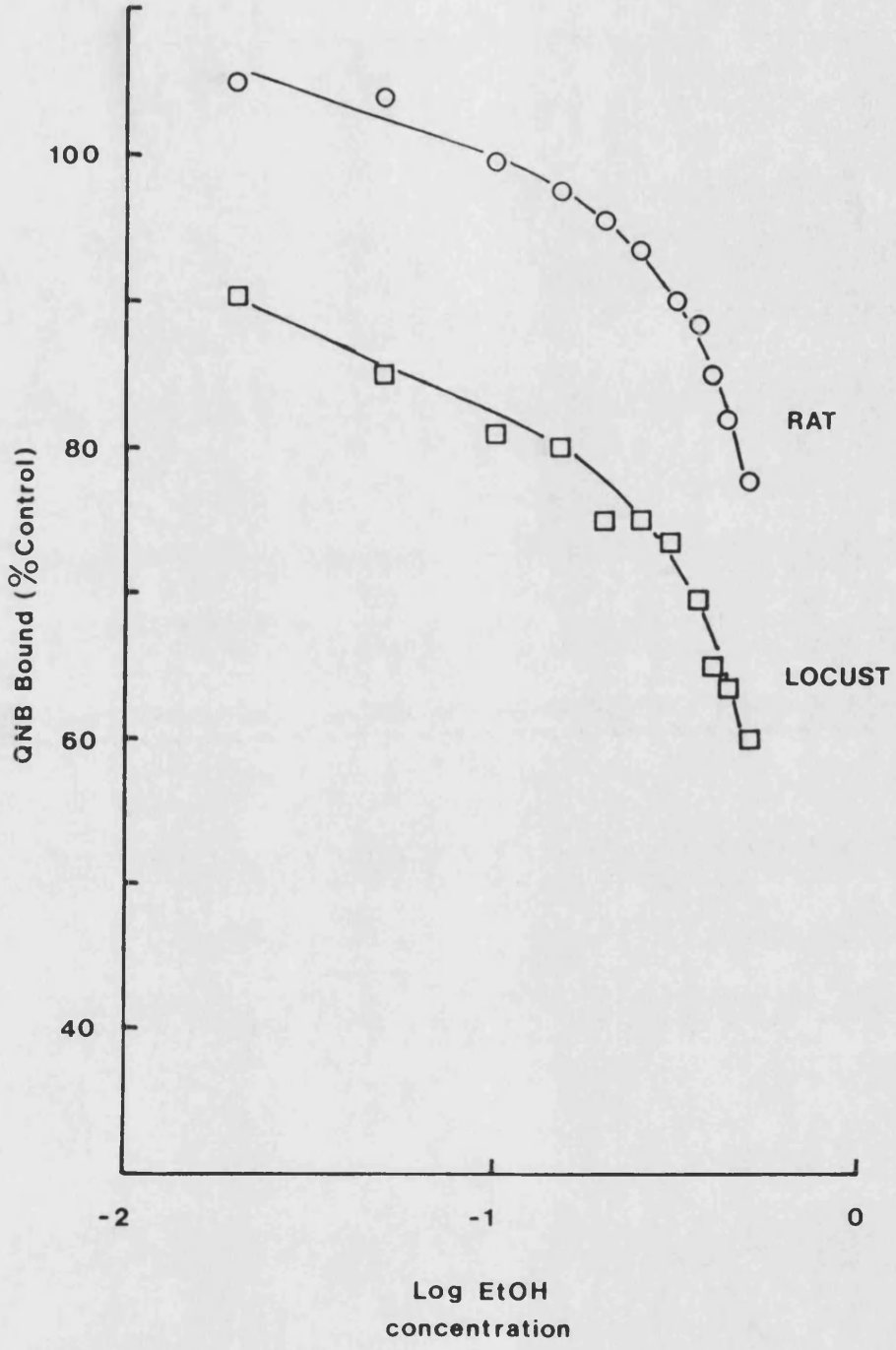

Figure 11. Effect of ethanol on [^{3}H]QNB binding to membranes.

Membrane samples prepared from either locust cerebral ganglia or rat cerebral cortex were incubated with the appropriate ethanol concentration at 25 °C for 1 h before the addition of [^{3}H]QNB to a final concentration of 1 nM. [^{3}H]QNB binding has been expressed as a percentage of that found for control membranes in the absence of ethanol. This is a representative result, one of three independent experiments.

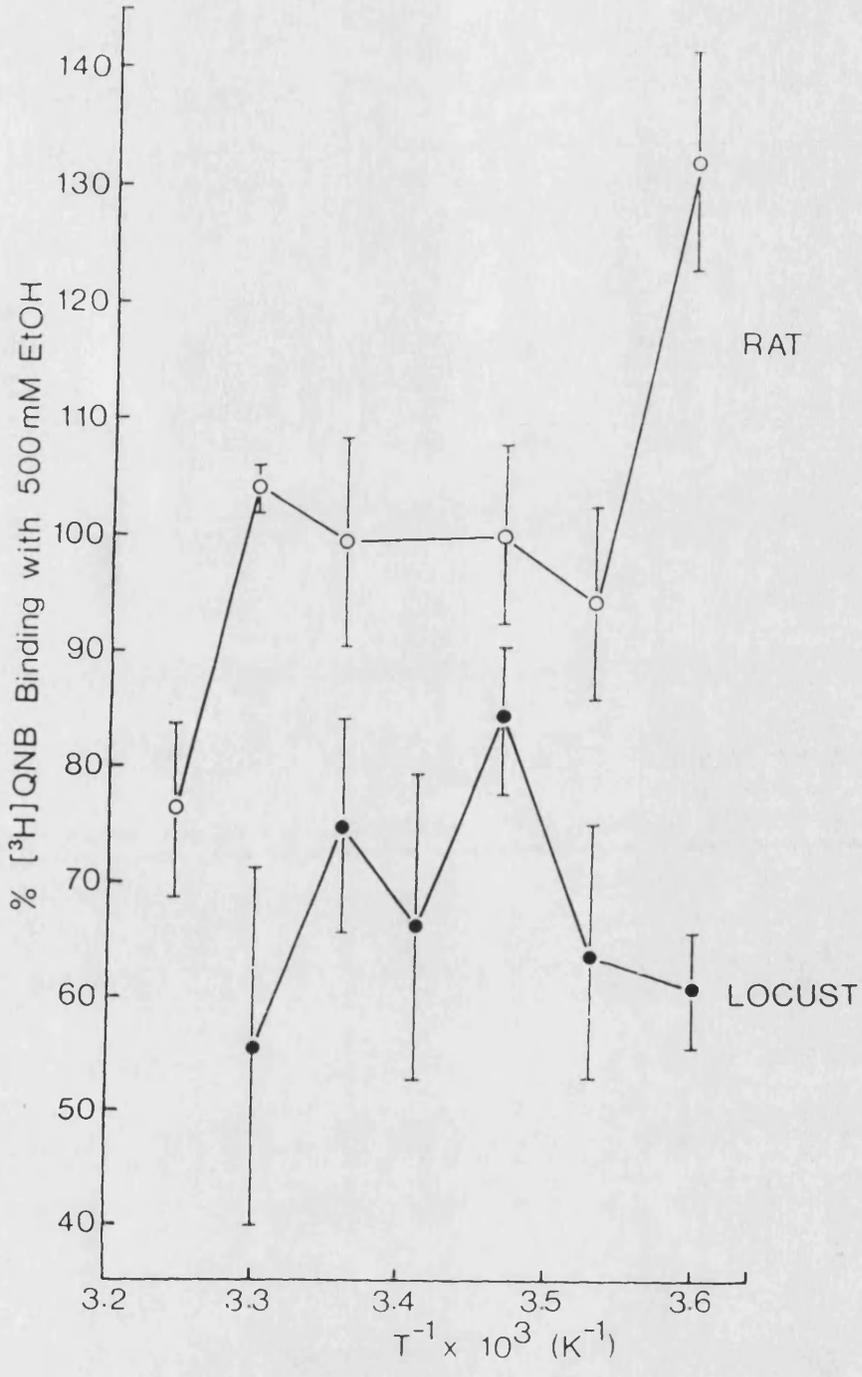

Figure 12. Pseudo-Arrhenius plot of ^{3}H]QNB binding to membranes in the presence of 500 mM ethanol.

Membrane samples prepared from either locust cerebral ganglia or rat cerebral cortex were incubated with 500 mM ethanol at the temperature specified for 1 h before the addition of [^{3}H]QNB to a final concentration of 1 nM. [^{3}H]QNB binding has been expressed as a percentage of that found for control membranes incubated at that temperature but in the absence of ethanol. The values are the means ± standard deviation from at least three independent experiments.

ethanol on the [^{3}H]QNB binding sites differs both quantitatively and qualitatively between the rat brain and locust ganglion membranes.

-Membrane anisotropy determined- -by fluorescence polarization-

4.4

The three fractions taken from the centrifugation described in section 4.1.1 were assayed for both protein concentration and [^{3}H]QNB binding. The results are shown in Table 5. Fraction 2, taken from the interface between the 0.25 M sucrose and the 1.2 M sucrose shows an enhanced concentration of specific binding sites.

4.4.1

The results of the fluorescence polarization measurements were calculated in terms of the fluorescence anistropy (r). This value for each temperature is calculated by the formula:

$$r = (I_{vv} - g.I_{vh}) / (I_{vv} + 2.g.I_{vh})$$

where I is the fluorescence intensity and the subscript indicates the orientation of the two polaroid filters on

the fluorimeter, v representing a vertical polarization and h a horizontal polarization. The first subscript refers to the filter between the source and the sample and the second to the filter between the sample and the detector. g is a correction factor for the geometry of the system and is equal to I_{vh} / I_{hh}.

Figure 13 is an Arrhenius plot of log r against T^{-1}, T is the thermodynamic temperature in Kelvins.

Fraction Number	Protein Recovered (mg)	Relative Specific Activity
1	1.2	0.9
2	0.4	5.2
3	3.3	0.5

Table 5. The subfractionation of a P_2 membrane fraction from locust cerebral ganglia.

This is one of two separate experiments, the relative specific activities in the other differed by less than 10% of the figures shown. The relative specific activities were calculated as the specific binding of [^{3}H]QNB (final concentration 2 nM) in cpm mg protein^{-1} of each fraction divided by the calculated specific activity of the whole.

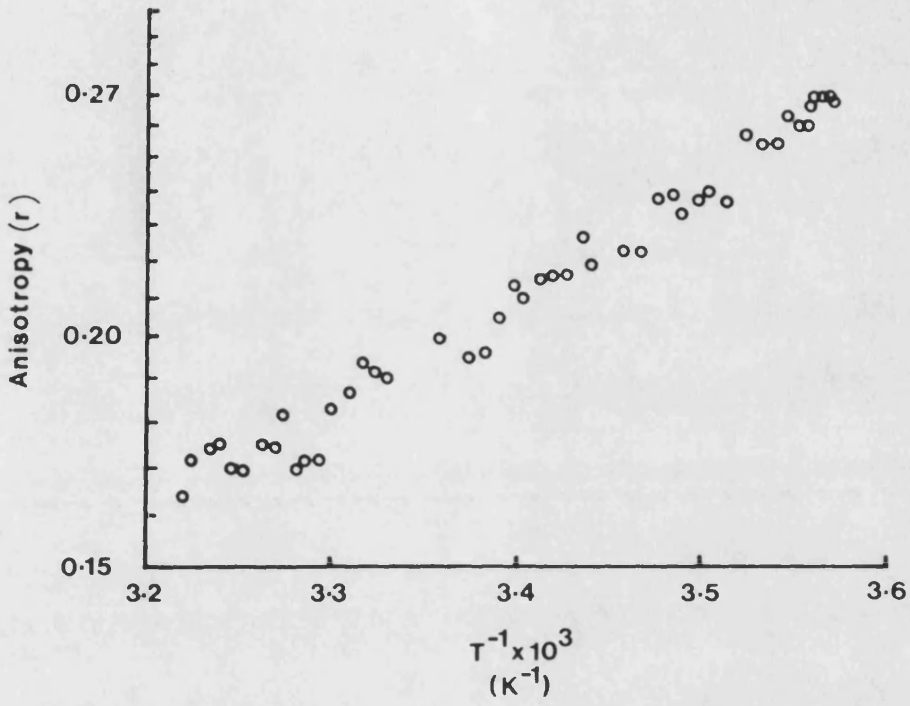

Figure 13. Arrhenius Plot of the anisotropy of locust ganglionic membranes.

This is one of two experiments. The anisotropy determined by the fluorescence polarization of diphenylhexatriene is plotted on a log scale.

-Physical properties of the membrane-
-and the muscarinic receptor-

4.5

Diacetal is a protein modifying reagent which is specific for arginine residues. Millan et al. (1984) used diacetal to determine whether an arginine residue might be important in ligand binding to the mAChR, but were unable to reach any firm conclusion about the site of action of diacetal although they did show that diacetal reduced the binding of [^{3}H]QNB to rat brain membranes.

If an arginine residue was important in the binding site for [^{3}H]QNB, then it was hypothesised that it might be conserved in evolution and perhaps experiments on the locust CNS might clarify the issue. In the locust diacetal was comparatively ineffective at reducing the binding of [^{3}H]QNB an effect only being observed at the highest concentration used by Millan et al. (1984). The decrease in [^{3}H]QNB binding did not follow the time course expected for a chemical reaction. Therefore experiments using the same concentration of acetone were performed in parallel to see if the observed effect was due to the solvent properties of the diacetal. Acetone ($CH_3.CO.CH_3$) is structurally similar to diacetal ($CH_3.CO.CO.CH_3$) but lacking the second carbonyl function necessary for the reaction with the arginine residues.

Acetone proved to produce a similar response to that given by diacetal, showing an initial increase in [^{3}H]QNB binding more marked than that shown by diacetal. This seemed to indicate that the effect observed was due to some physico-chemical property of these compounds which might involve their attributes as organic solvents.

Ethanol is an organic solvent that has effects on both receptors (Tabakoff and Hoffman, 1983) and membranes (Chin and Goldstein, 1981). These have been well studied due to the interest in establishing the cause of alcohol induced intoxication and the mechanism of addiction. Aguilar et al. (1984) had already shown that ethanol was more potent at reducing the binding of [^{3}H]QNB to locust preparations than to rat brain membranes, and this was confirmed. To see if this effect was due to a membrane fluidity change the effect of temperature on the inhibition of [^{3}H]QNB binding was investigated. If the effect was physical in nature rather than chemical it was anticipated that an Arrhenius plot of the data would show an inflexion or dicontinuity which might correspond to a phase change in the lipid environment of the receptor.

The results of these experiments showed that the curves for rat brain and locust ganglion membranes were very different in shape. The curve for the rat brain membrane experiments follows the form anticipated. At low temperatures (5^{o}C) ethanol (500 mM) actually

enhances binding over control, perhaps by fluidizing a membrane which has entered the gel phase. As the temperature is increased the binding falls to a stable level not significantly different from control values and at more physiological temperatures (35°C) the binding is inhibited by ethanol, perhaps indicating an unusual fluidity state, or 'membrane expansion' which has been suggested to explain the efect of ethanol on membranes (Chin and Goldstein, 1981).

In the locust the greatest inhibition by ethanol is again seen at the highest temperature (30°C) at which point the binding was only 56% of the control value, which is above the temperature at which the locusts were kept before being killed (25°C). The inhibition of [^{3}H]QNB binding decreases as the temperature is decreased but after reaching a minimum at 15°C at which point the binding was 83% of the control value the binding in the presence of ethanol falls off sharply as the temperature is further decreased. This fall at lower temperatures is difficult to explain, if membrane fluidity was the sole mechanism of effect then it would be expected that the binding of [^{3}H]QNB in the presence of ethanol would continue to rise as the temperature fell, as in the rat. It must therefore be postulated that there is either some unique feature of locust ganglionic membranes that causes them to respond differently to ethanol at low temperatures or that the ethanol is having an effect directly on the receptor.

To determine whether the effect of ethanol might have been due to some major difference in the properties of the membrane, it was first necessary to determine if the physical properties of the insect neuronal membrane, about which nothing was known, were different from those of the rat neuronal membrane, which have been characterized.

A standard measure of the physical properties of biological membranes is the rotational viscosity. There are several methods of measuring this by probing the membrane. The one used in this study, fluorescence polarization is one of the most commonly used (for review see Shinitzy and Barenholz, 1978). It relies on the properties of the probe diphenylhexatriene (DPH), which is a hydrophobic compound which readily penetrates the membrane and when in a hydrophobic enviroment is capable of fluorescing.

The polarization of the locust membranes is of the same order as that found for mouse synaptosomal membranes in the study by Harris and Schroeder (1981). At a temperature of 30^{o}C Harris and Schroeder (1981) found a polarization of DPH of approximately 0.28 whereas in this study the polarization of DPH was 0.255 at 29.7^{o}C. There is, however a significant difference in the results from Harris and Schroeder (1981), they observed a discontinuity in the Arrhenius plot of the polarization of DPH, at 23^{o}C, indicating a phase change in the lipid at this temperature. In contrast there is

no detectable discontinuity in the Arrhenius plot of the fluorescence anisotropy from the locust.

Discontinuities in arrhenius plots are not always observed even in mammalian membranes (Shinitzy and Barenholz, 1978). It is thought that high concentrations of cholesterol can cause loss of phase changes in membranes. Thus it may be that the the higher proportion of cholesterol found in the membrane preparation analyzed in these studies, as compared with that found in the rat synaptosomal preparation of Breckenridge et al. (1972), may be of some importance in the observations of the physical properties in this study.

The phase changes indicated by discontinuities are thought to be a transition between a fluid state of the membrane and a gel state. A gel state implies a rigid structure where not only is the rotational viscosity markedly higher but also the translational viscosity. This would make it much more difficult for movement to occur in the plane of the membrane. There are several processes in the nervous system which are dependent on components of the membrane moving in the plane of the membrane, these include the events of the control of second messenger systems where some combination of the receptor, the N protein and the effector enzyme must move through the membrane lipid in order to interact. Thus a gel phase must be avoided in the physiological temperature range of an animal. The

phase change seen by Harris and Schroeder (1981) at 23°C is well below the physiological temperature of the rat, and so is without consequence. In contrast, for the locusts in this study, which were kept at 25°C, a phase change at 23°C would be quite unacceptable. The locusts were seen to still be active, albeit sluggishly, at temperatures well below that, when being anaesthetized with dry ice, and when dry ice was not available the locusts were chilled, in order to render them easy to handle, they were still capable of movement and some response to their surroundings at 4°C. Furthermore locusts which had been anaesthetised with dry ice and frozen were capable, if allowed, of reviving.

Thus locusts are capable of surviving great changes in temperature which occur very rapidly. In the time scale of, at most, a few minutes it is unlikely that the lipid composition of the membranes can change to any great degree and therefore it would seem that the ability to remain active throughout such temperature changes is inbuilt rather than an adaptive mechanism.

It would seem, therefore, that the differences in membrane viscosity between the neuronal membrane of the rat and the locust reflect the major difference in their physiology.

-Further work-

4.6

The neuronal membrane of insects has never previously been studied in this fashion, there are differences between it and the membrane of the rat neurone and these may well be worth investigating, particularly with respect to the function of the neurotranmitter receptors, and other membrane proteins. This is of interest because many of the insecticides in common use are highly hydrophobic, although they are mainly thought to affect ion channels their effects seem to be wide ranging and given the poor solubility of many of them, such as lindane (hexachlorocyclohexane), in aqueous solution it would seem likely that they would pass freely into membranes. Thus it may be that the classes of insecticide such as the cyclodienes and lindane which are hydrophobic, may act through binding to membrane lipid sites on the proteins on which they act or by otherwise disrupting the protein lipid interactions. Studies on the effect of insecticides on membrane fluidity might be very rewarding.

In practice these sorts of experiments should not be very difficult, they have the advantage that they require comparatively little tissue, always a limiting factor in insect research and the techniques are readily transposable to insect tissue.

CHAPTER 5.

MUSCARINIC RECEPTOR HETEROGENEITY

-Measurement of phospholipid turnover-

5.1

Supraoesophageal ganglia, from locusts not treated with carbon dioxide, were very carefully dissected to remove the optic lobes. The ganglia were then cut across once with a scalpel blade and placed immediately into oxygenated locust saline containing 10 mM glucose. For each of the four samples 20 ganglia were placed in 10 ml of saline A.

The ganglia were incubated with 100μCi of ^{32}P (as $H_3 PO_4$, carrier free, Amersham International plc) for 30 min at room temperature.

The samples were incubated for 20 min at 25°C to allow the samples to equilibrate before the addition of 100-150 μCi of carrier free ^{32}P to each sample. Carbamoyl choline (final concentration 10 mM) was immediately added to two of the samples. The samples were then incubated at 25°C for 60 min.

The reaction was stopped by centrifuging the tubes (10 min, 500 xg), pouring off the supernatant and adding 10 ml chloroform:methanol (2:1, by volume) to the ganglia.

The phospholipids were extracted by a modified 'Folch' extraction as described above (section 3.1).

-Separation of phospholipid classes-

5.1.1

The neutral lipids were removed from the total lipid extract obtained from the Folch extraction by washing a dry lipid film with acetone; neutral lipids are soluble in acetone whereas phospholipids are not (Lovelock et al., 1960). A more rigorous separation of the different phospholipid classes was required than that given by the method of Skipski et al. (1964) given above (section 3.2). Consequently a two-dimensional separation by thin layer chromatography was employed (Abdel-Latif et al., 1974).

In this separation glass plates (20 cm x 20 cm) were spread with a slurry of 20g silica gel H 'nach Stahl' in 65 ml of water containing 1.5 g of magnesium acetate. The plates were allowed to dry, and then activated for at least an hour in an oven at 100°C before use.

A sample of phospholipid was applied to a corner of each plate. The plates were developed in the first direction with $CHCl_3$: CH_3OH:28% NH_3 (aq.) (65:25:4, by volume) Then the plate was removed from the tank and dried with a hair-dryer, before being reactivated in an oven at 100°C for 1 h.

The plates were developed in the second direction with C_4H_9OH: CH_3CO_2H:H_2O (120:20:20, by volume)

The phospholipids were visualised by exposure

to iodine vapour. The spots were then scraped onto glazed paper and the silica transferred to glass centrifuge tubes, which contained 3 ml of the developing solvent from the method of Skipski et al. (1964) (see section 3.2). The lipids were extracted by mixing the silica with the solvents given below in that order and then centrifuging (10 min 500 xg) and collecting the solvent. The solvents employed were:

1. 3 ml of Skipski developing solvent
2. 2 ml of Skipski developing solvent
3. 2 ml of CH_3OH
4. 2 ml of CH_3OH: CH_3 CO_2H: H_2O (94:1:5, by volume)

-Determination of the specific activity-

5.1.2

The samples were evaporated to dryness in a boiling water-bath in boiling tubes with an anti-bumping granule. They were then ashed to give inorganic phosphate and the phosphate concentration assayed according to the method of Bartlett (1959).

In this method 1.0 ml of water and 0.5 ml of 5M sulphuric acid were added to the samples and the resulting mixture heated in an oven at 150°C overnight. The following day 2 drops of hydrogen peroxide (30%) were added and the solution was returned to the oven (160°C) for at least 1.5 h more to complete the

combustion and to decompose the remaining hydrogen peroxide.

4.4 ml of water was added to the tubes the solution was mixed and 4.4 ml transferred to scintillation vials and the activity measured using the Cherenkof radiation, in a liquid scintillation counter set for reading tritium.

4.3 ml was transferred from the scintillation vials into glass test tubes, to which were added 0.2 ml ammonium tetramolybdate (5%, w/v) and 0.2 ml of Fiske-SubbaRow reagent. The Fiske-SubbaRow reagent was prepared by adding 0.5 g of 1-amino-2-naphthol-4-sulphonic acid to 200 ml of freshly prepared 16% (w/v) sodium metabisulphite followed by 1.0 g of anhydrous sodium sulphite. The solution was filtered and used the same day.

The samples were returned to the oven (110^{o}C) for 15 min, then removed and allowed to cool to room temperature before reading the absorbance at 830 nm. The phosphate content was determined by comparison with a standard curve prepared from inorganic phosphate.

The ratio of the activity of the phospholipid samples to the mass of phosphorus is the specific activity. The specific activity is a measure of the turnover rate for the phospholipids.

-The incorporation of [^{3}H]inositol- -into the phospholipid-

5.2

Supraoesophageal ganglia were carefully dissected and sliced, as before, and immediately placed in gassed (O_2: CO_2; 95: 5) saline B. About 10 ganglia were used per sample in 10ml of saline.

The dissected ganglia were allowed to equilibrate with the buffer for 1 h at room temperature. Then the saline was changed to 2 ml fresh gassed saline and 2µCi of myo [2-^{3}H]inositol (10-20Ci mmol^{-1}, 1mCi ml^{-1}, supplied by Amersham International plc) were added in the presence or absence of 0.1 mM carbachol and other cholinergic drugs. The incubation was allowed to continue for 1 h. The incubation was stopped by centrifuging the tubes to pellet the ganglia and decanting the supernatant, 5 ml of chloroform: methanol (2:1) were added and the ganglia were rapidly homogenised (10 s, Ultraturrax). The lipids were extracted by the method given above (section 3.1).

The lipid samples were dissolved in 5 ml $CHCl_3$: CH_3OH (2:1, by volume) and divided, 4 ml transferred to a scintillation vial and dried before the addition of 5 ml of 'Optiphase Safe' scintillant for liquid scintillation counting and the remainder ashed and assayed for the inorganic phosphate content (Bartlett, 1959. See section 5.1).

Saline A, used in the measurement of phospholipid turnover.

NaCl,	150 mM
KCl,	10 mM
$CaCl_2$,	2 mM
$MgCl_2$,	5 mM
'Tris',	10 mM
glucose,	10 mM

Made to pH 6.8 with 36% (v/v) hydrochloric acid.

Saline B, used in the incorporation of [^{3}H]-inositol.

NaCl,	150 mM
KCl,	10 mM
$CaCl_2$,	2 mM
$MgCl_2$,	5 mM
$NaHCO_3$,	8 mM
KH_2PO_4,	6 mM
glucose,	10 mM

pH 6.8

-Determination of cAMP production-

5.3

Supraoesophageal ganglia were dissected from freshly decapitated locust heads and immediatey frozen with liquid nitrogen before storing at -80°C. Adenylate cyclase was assayed in homogenates of these ganglia. Ten ganglia were homogenized in 0.5 ml of 50mM Tris buffer (pH 7.5) containing $MgCl_2$, 6 mM; NaCl, 90 mM; EDTA, 0.8 mM; theophylline, 8 mM. The homogenate was passed through nylon mesh to remove large tissue fragments and the volume made up to 2 ml.

The assay was carried out in the same buffer, with the addition of ATP (1 mM), GTP (0.1 mM) and cholinergic ligands.

A total volume of 0.5 ml was used per assay tube including 0.05 ml of homogenate. The reaction was started by the addition of the ATP and GTP and stopped by placing the tubes in boiling water for 5 min. Protein was precipitated by centrifugation, and the cAMP concentrations determined in a competition assay with a cAMP binding protein and [^{3}H]cAMP (Amersham International) as desribed by Brown *et al.*(1971) with cAMP binding protein fom BDH Ltd.

-Incorporation of ^{32}P into the membrane phospholipids-

5.4

The two dimensional thin-layer chromatogram developed according to Abdel-Latif et al. (1974) provided a much better separation of the important classes of phospholipid (PI and phosphatidic acid) than the one dimensional system of Skipski et al. (1964) used before (see Fig. 14).

As less tissue was used in these experiments than in the compositional analysis, preliminary experiments showed that the phosphate assay system used before was not sufficiently sensitive, particulary for the assay of phosphatidic acid which was present in very small quantities. This was due to the dry ashing procedure which used large amounts of acid, which interfere with the colour reaction. The procedure of Bartlett (1959), employed as suggested for microdetermination, with wet ashing was found to be much more sensitive (see Fig. 15).

The presence of carbachol (10 mM final concentration) did not have any apparent effect on the incorporation of ^{32}P into any of the phospholipid classes. It was possible, however, to measure the specific incorporation of ^{32}P into the different phospholipid classes which is a measure of their respective turnover rates (see Table 6).

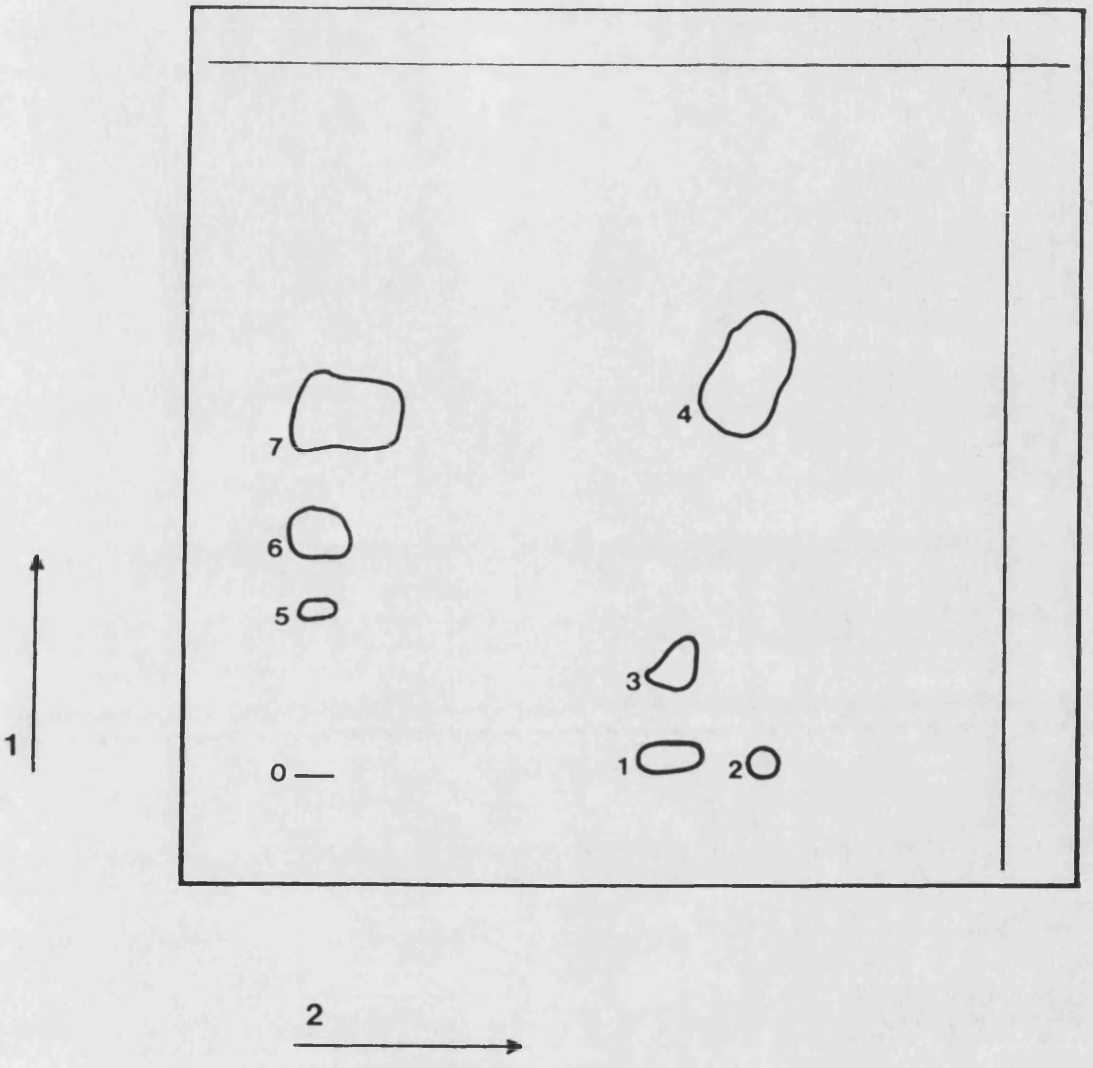

Figure 14. Two dimensional thin-layer chromatogram of phospholipids by the method of Abdel-Latif et al. (1974).

The phospholipids were visualised by exposure to iodine vapour. 0. Origin; 1. Phosphatidylserine; 2. Phosphatidic acid; 3. Phosphatidylinositol; 4. Phosphatidylethanolamine; 5. Lysophosphatidylcholine; 6. Sphingomyelin; 7. Phosphatidylcholine.

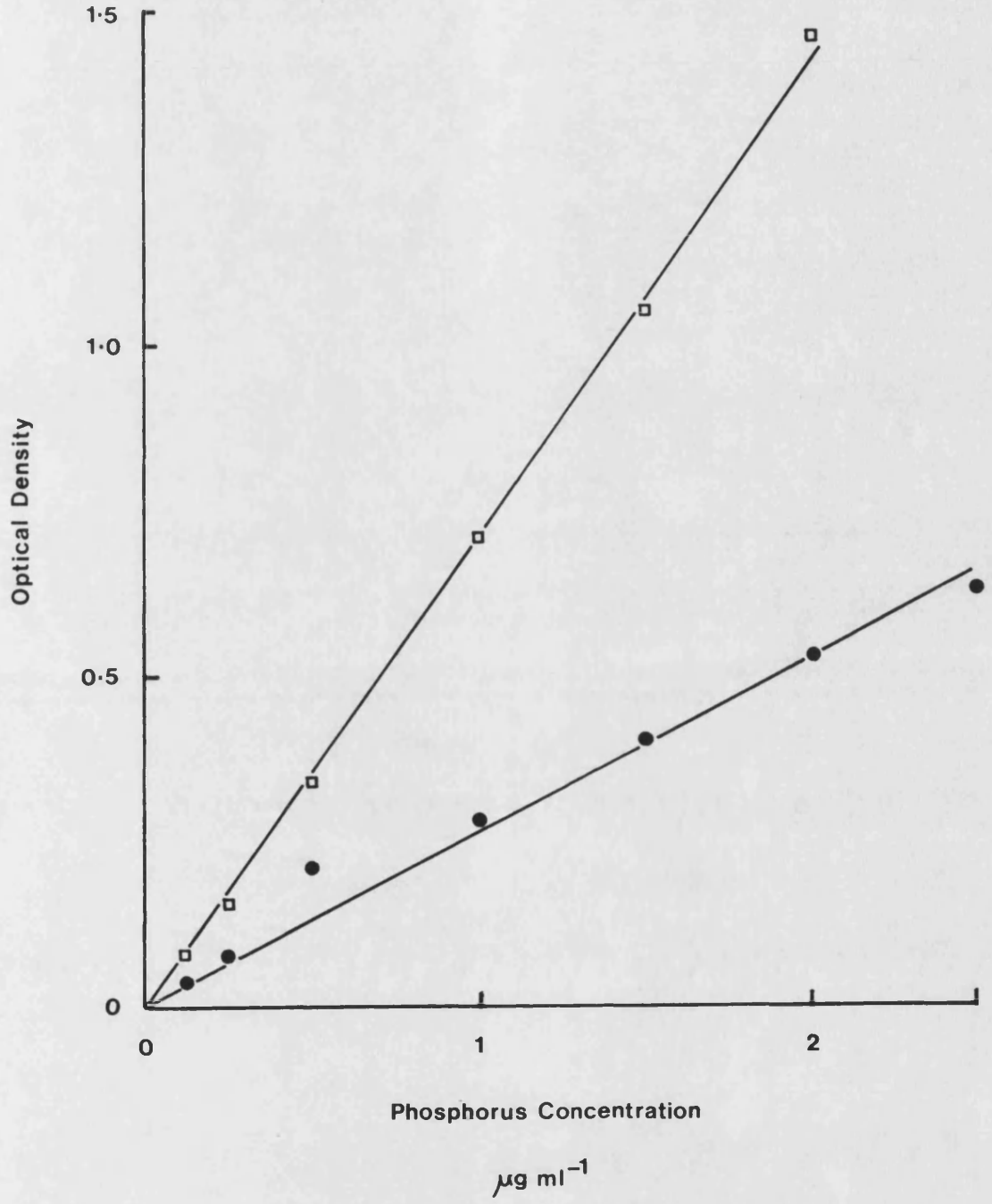

Figure 15. Comparison of two methods for the determination of phosphorus concentration.

The two methods used were those given in sections 3.3 (●) and 5.1.2 (□). These are typical results for the standard curves, in each case the phosphorus concentrations were made up using disodium hydrogen orthophosphate. Optical densities were measured against reagent blanks in both cases.

Phospholipid	^{32}P Incorporation.
Phosphatidic Acid	7.35 ± 3.46
Phosphatidylserine	0.78 ± 0.44
Phosphatidylinositol	11.63 ± 1.52
Phosphatidylethanolamine	0.25 ± 0.14
Phosphatidylcholine	0.23 ± 0.15

Table 6. Incorporation of ^{32}P into the phospholipid classes of locust cerebral ganglia.

Results are expressed as the ratio of the specific activity (in cpm μg^{-1} P) of the individual phospholipid class (after 1 h in saline containing 10 µCi ^{32}P ml^{-1}) to that of the total phospholipid fraction from each of 6 experiments. Errors are one standard deviation from the means.

-Incorporation of [^{3}H]inositol- -into the phospholipids-

5.5

The incorporation of [^{3}H]inositol was studied as a more convenient means of determining the effect of ligands on PI turnover than the incorporation of ^{32}P.

The [^{3}H]inositol was incorporated entirely into the PI fraction of the phospholipid. This was shown in an experiment in which the lipid extract was subjected to the same separation procedure as that used in the ^{32}P incorporation experiments (section 5.1.1), and the separate fractions mixed with scintillation fluid and counted by liquid scintillation counting. No fraction other than that corresponding to PI showed activity above background.

The time course of [^{3}H]inositol incorporation was followed for up to one hour. The incorporation of [^{3}H]inositol into the lipid fraction had not saturated after one hour as shown in Figure 16. The rate of uptake was nearly linear.

Preliminary experiments in which the 1 h equilibration period was not employed showed no effect of carbachol and, furthermore, gave variable control incorporation (see Table 7).

Results obtained after the inclusion of a 1 h equilibration period in the method showed significantly

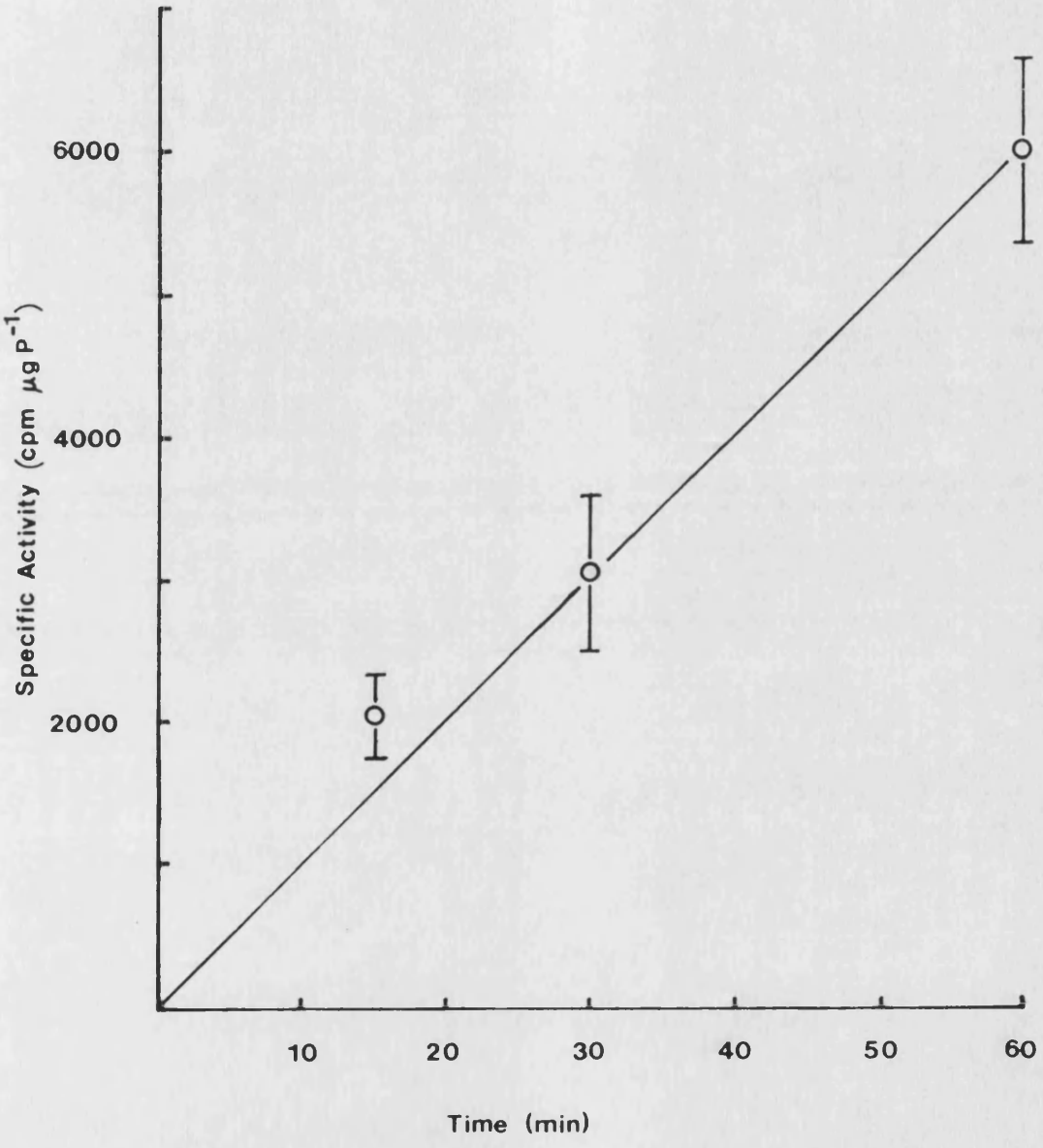

Figure 16. Time course of the incorporation of [^{3}H]inositol into the ganglionic lipids.

The result shown is one of two experiments. The values are the mean and spread of duplicate determiations. These experiments were performed with a 1 h preincubation period before the addition of the [^{3}H]inositol. The incorporation was calculated as cpm per µg total lipid phosphorus.

greater incorporation of label in the presence of 1 mM carbachol, which response was blocked by the antagonists atropine and pirenzepine (see Table 8).

Other workers have had difficulty in showing agonist stimulation of PtdIns turnover in insect central nervous systems (Ross and Brady, 1986; Trimmer and Berridge, 1985). Trimmer and Berridge (1985) suggested that this might be due to high levels of endogenous neurotransmitter release; it may be that the equilibration period used is sufficient for endogenous neurotransmitter concentrations to decrease or for the ganglia to return to a resting state.

Ligand	Specific Activity. (cpm μg P^{-1})
None	2243 ± 1690
Carbachol (10^{-3} M)	3809 ± 489
Atropine (5×10^{-5} M)	3953 ± 1531

Table 7. The incorporation of [^{3}H]inositol into the phospholipid pool of freshly dissected locust cerebral ganglia.

The values shown are the result of a typical experiment (one of 8) showing incorporation of [^{3}H]inositol into locust cerebral ganglia. These experiments were performed without any preincubation before the addition of the [^{3}H]inositol and specified ligands. The specific activities shown are the means of triplicate values and are expressed ± one standard deviation.

Ligand	Specific activity (% of control)	n
None	100 ± 16	8
Carbachol (10^{-3}M)	164 ± 22	6
Carbachol (10^{-3}M) & Atropine (5×10^{-5}M)	91 ± 21	4
Carbachol (10^{-3}M) & Pirenzepine (5×10^{-6}M)	105 ± 25	2

Table 8. The effect of cholinergic ligands on the incorporation of [^{3}H]inositol into the phospholipid pool.

The values are the means of results from n separate experiments, calculated as the specific activity of the phospholipid (cpm μg P^{-1}) and expressed as a percentage of the value for the control sample. The errors shown are the standard deviations from the means. All these experiments were performed including a 1 h preincubation before the addition of [^{3}H]inositol and the specified ligands.

-Production of cAMP-

5.6

The assay procedure outlined (section 5.3) showed that the production of cAMP was linear for a period of 10 min (see Figs. 17 and 18). The values for the cAMP produced during the first 5 min can be used to calculate an apparent adenylate cyclase activity for the ganglionic homogenate of 205 $\pm$ 17 pmol cAMP.min^{-1}.mg $protein^{-1}$.

The activity of the adenylate cyclase was reduced by carbachol in the presence (Fig. 17) but not in the absence (Fig. 18) of 100 mM sodium chloride. The reduction of cAMP production by carbachol was dose-dependent (see Fig. 19). The muscarinic antagonist atropine prevented the down-regulation of cAMP production by carbachol (see Table 9).

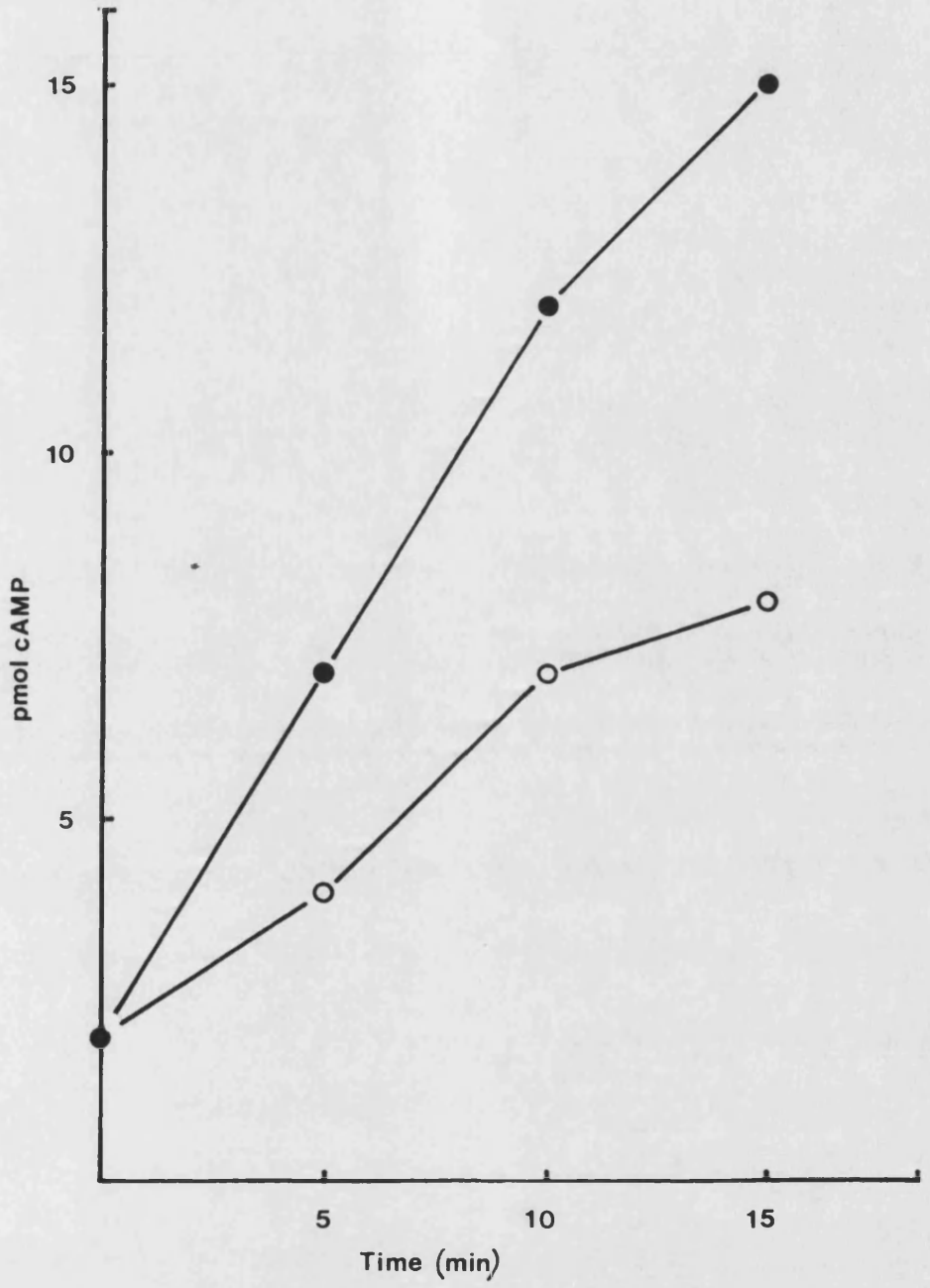

Figure 17. Time course of cAMP production; Effect of carbachol in the presence of 90 mM sodium chloride.

This is a representative experiment, one of four. The values are the means of duplicate determinations of cAMP concentration, in the presence (O) or absence (●) of 10mM carbachol. The experiments were performed according to the method given in section 5.3.

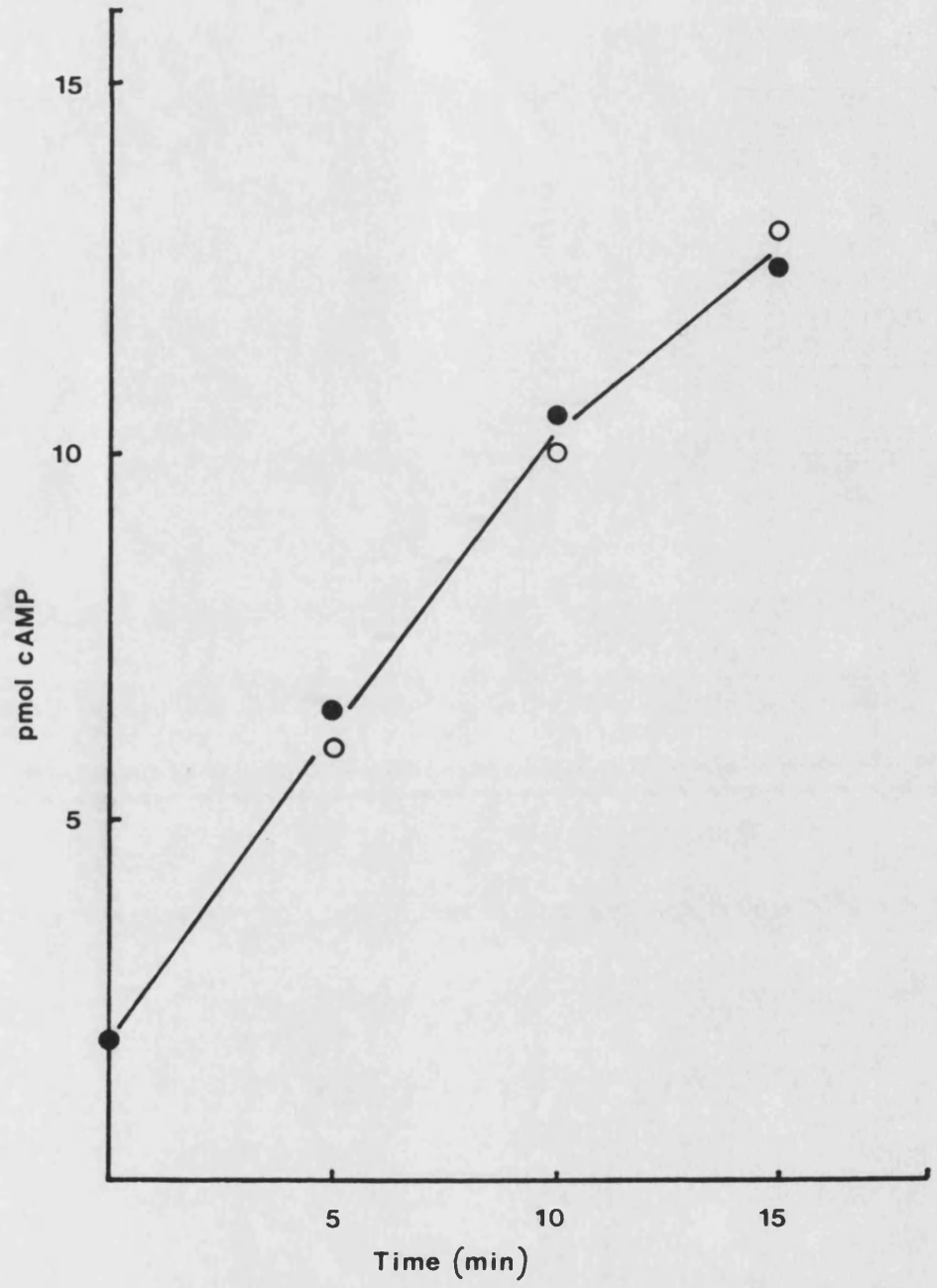

Figure 18. Time course of cAMP production; Effect of carbachol in the absence of sodium chloride.

This is a representative experiment, one of three. The values are the means of duplicate determinations of cAMP concentration, in the presence (O) or absence (●) of 10mM carbachol. The experiments were performed according to the method given in section 5.3 except that the buffer used contained no sodium chloride.

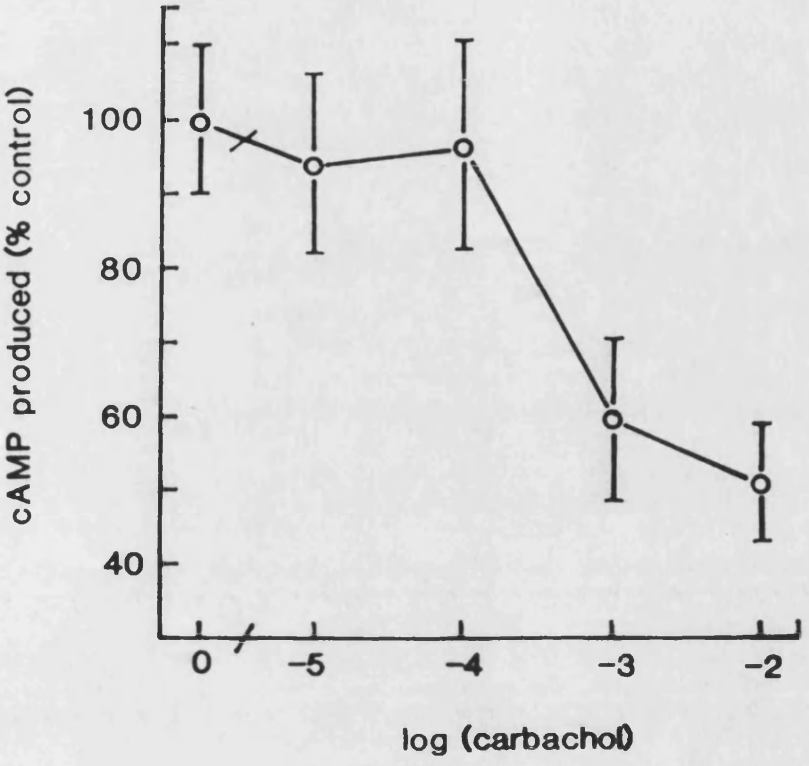

Figure 19. Effect of carbachol concentration on cAMP production.

The amount of cAMP produced by the ganglionic homogenate in 5 min was determined as a measure of the adenylate cyclase activity. The effect of the inclusion of a range of concentrations of carbachol in the reaction buffer is shown. The values are expressed as a percentage of the concentration of cAMP produced by the ganglionic homogenate in the absence of carbachol and represent the mean $\pm$ standard deviation of three independent experiments, in each of which duplicate determinations of cAMP concentration were made.

Ligand	cAMP production (% of control)	n
None	100 ± 10	4
Carbachol (10^{-2}M)	51 ± 8	4
Carbachol (10^{-2}M) & Atropine (10^{-5}M)	75 ± 4	4

Table 9. Regulation of cAMP production.
Results are expressed as mean ± standard deviation of the % of the cAMP produced by the control in each of n separate experiments.

-Inhibition of [^{3}H]QNB binding by- -selective muscarinic ligands-

5.7

To express the affinity of an unlabelled ligand for a binding site, the IC_{50} or K_i value is used. The IC_{50} value is the concentration of that ligand required to displace 50% of the maximum specific binding of a standard radiolabelled ligand, in this case [^{3}H]QNB. When this displacement is competitive the value is dependent on the concentration of radiolabelled ligand. To allow for this the potencies are better expressed as K_i values, as these are independent of the concentration of radiolabelled ligand used. K_i values are calculated from the IC_{50} values by the formula:

$$K_i = IC_{50} / (1 + L / K_D)$$

Where L is the concentration of radiolabelled ligand used and K_D is the dissociation constant for that ligand.

[^{3}H]QNB binding to the ganglionic P_2 fraction generaly gave triplicate values with a standard deviation from the mean of less than 7%. The non-specific binding (assumed to be that found when the assay was performed in the presence of 1 mM atropine) was always less than 20% of the binding in the absence of any competing ligand. The maximum specific binding was taken to be the difference between these two values. The results were expressed as a % of this figure.

For the determination of IC_{50} values for unlabelled ligands, the [^{3}H]QNB concentration used was 1 nM. The IC_{50} concentrations were initially determined from the data displayed as a Hill Plot. In this plot of $\log(B / (B_{max} - B))$ against log I the IC_{50} value is given by the intercept at the x axis.

The Hill Plot is also useful because the slope of the line gives an indication of deviations from the Law of Mass Action. If the Law of Mass Action is followed the Hill Plot of the data will have a slope of unity. Deviations can take the form of a slope greater than unity, corresponding to positive cooperativity, or a slope less than unity which indicates either negative cooperativity or the existence of two noncooperative sites with different affinities for the ligand.

The plots of the inhibition of [^{3}H]QNB binding are shown (see Figs. 20, 21, 22), and the data derived from them (see Table 10)

-Computer fitting of data-

5.7.1

Although the Hill plot is useful in detecting the possibility of binding site heterogeneity it does not give any quantitative information of the affinity or proportion of the different sites. For determining these parameters the best method is computer aided curve fitting of the data.

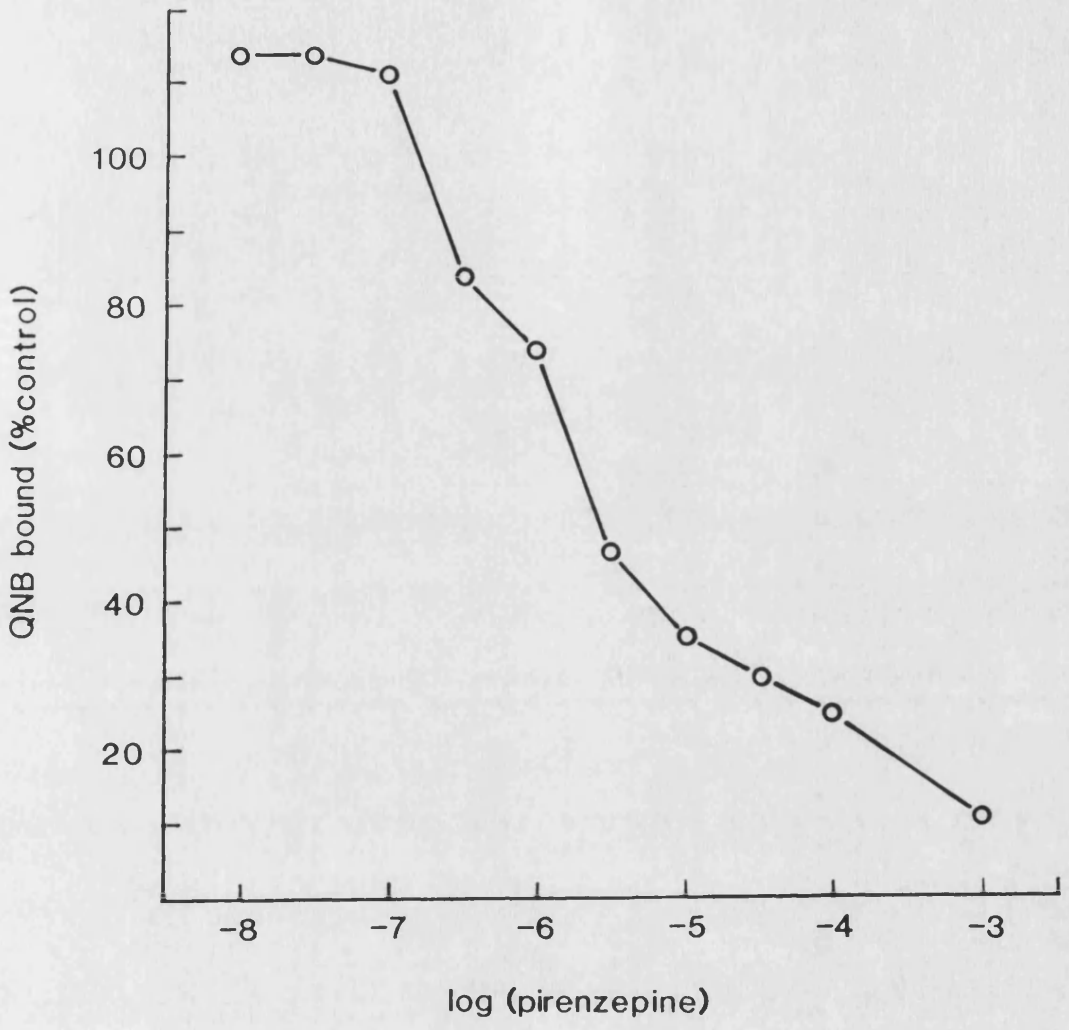

Figure 20. Inhibition of [^{3}H]QNB binding by pirenzepine.

This is a representative experiment, one of four. The specific binding of 1 nM [^{3}H]QNB is expressed as a percentage of the specific binding in the absence of any competing ligand. All points are the results of triplicate determinations, the standard deviation was always less than 7%.

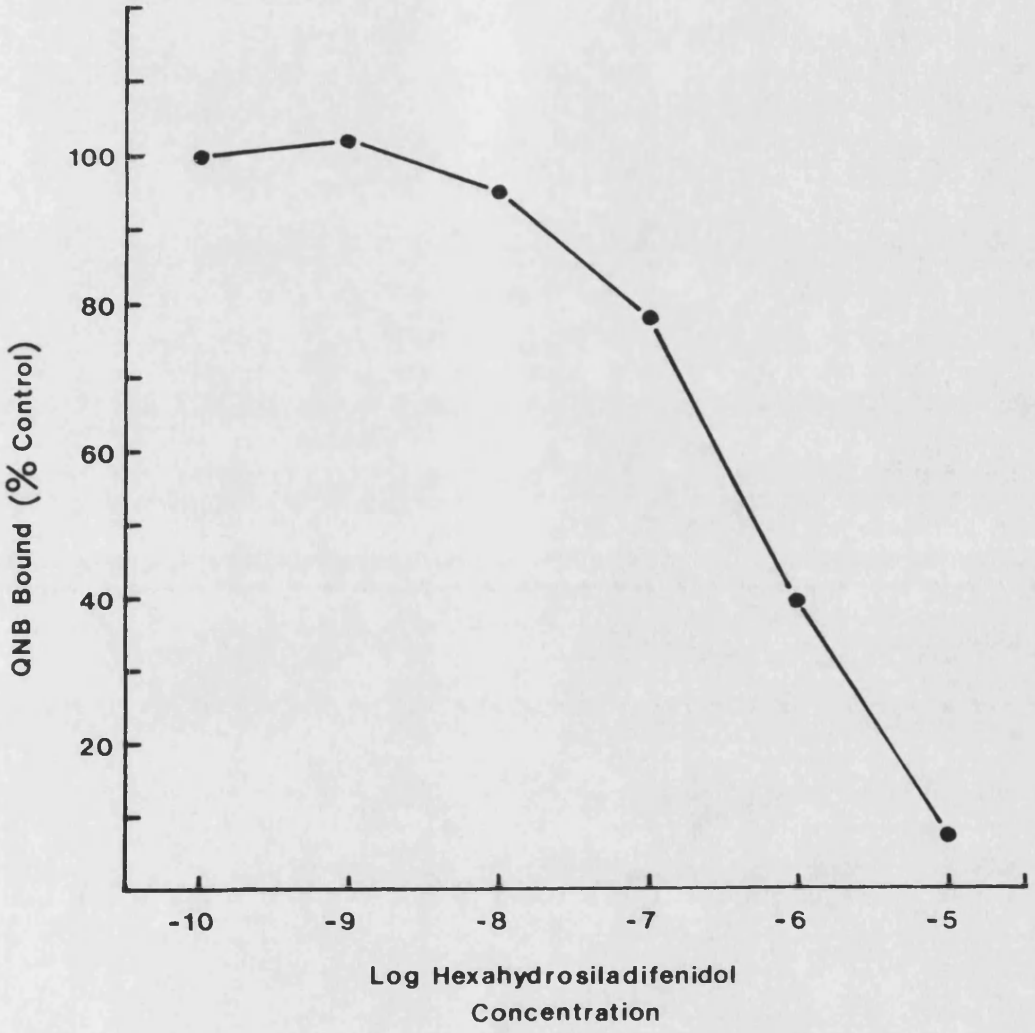

Figure 21. Inhibition of [^{3}H]QNB binding by hexahydrosiladifenidol.

This is a representative experiment, one of three. The specific binding of 1 nM [^{3}H]QNB is expressed as a percentage of the specific binding in the absence of any competing ligand. All points are the results of triplicate determinations, the standard deviation was always less than 7%.

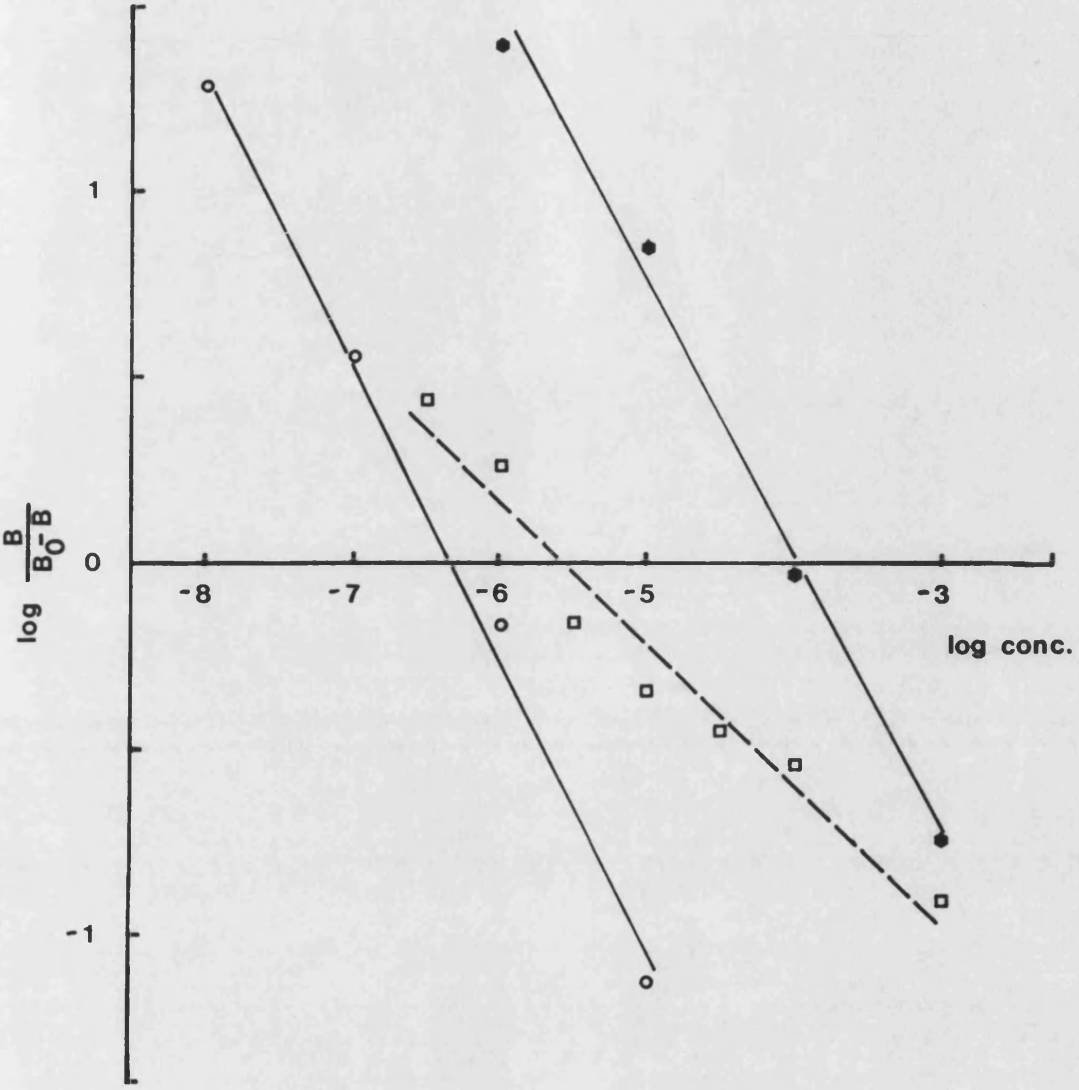

Figure 22. Hill Plot of the inhibition of [^{3}H]QNB by selective muscarinic ligands.

These plots are all conversions of typical experiments. Pirenzepine (□– –□). Hexahydrosiladifenidol (o——o). AF DX 116 (●——●)

The data from the inhibition of [^{3}H]QNB was modelled using the "By Hand" program of Richardson and Humrich (1983) modified for use on a BBC microcomputer. This program analyses the "goodness of fit" of operator generated models to the % occupancy curve for the test ligand.

A two-site model gave a statistically better fit, determined by a partial F test, than a one site model. When corrected for the concentration of [^{3}H]QNB the affinities and proportions of the two sites are:

K_{i1} 0.15×10^{-6} M - 55%

K_{i2} 7.5×10^{-6} M - 60%

The proportions of the two sites are expressed as % of the binding in the absence of any pirenzepine. However the best fit was obtained using as the maximum binding the [^{3}H]QNB bound in the presence of 10 nM pirenzepine which was, in all experiments, about 115% of the control value.

Ligand	K_i (μM)	N_H
Pirenzepine	1.9 ± 1.1	0.50 ± 0.04
Hexahydrosiladifenidol	0.24 ± 0.12	0.65 ± 0.18
AF DX-116	52 ± 34	0.63 ± 0.21

Table 10. Inhibition constants (K_i) and Hill Numbers (N_H) for the inhibition of [^{3}H]QNB binding by selective muscarinic ligands. Each value is the mean for three separate experiments and is given ± one standard deviation.

-Second messengers linked to the mAChR-

5.8

-Adenylate cyclase regulation-

5.8.1

Although an adenylate cyclase activity had previously been shown in the cerebral ganglion of Schistocerca gregaria (Morton, 1984) it had not been shown to be modulated by a mAChR. The only published use of cholinergic ligands in an insect CNS adenylate cyclase assay is that of Suter (1986) but the omission of Na^+, required in the mammalian system, lead to inconclusive results.

The apparent adenylate cyclase activity found in this study (205 $\pm$ 17 pmol cAMP.min^{-1}.mg $protein^{-1}$) agrees closely with the result of Morton (1984; 209 $\pm$ 13 pmol cAMP.min^{-1}.mg $protein^{-1}$), for the same tissue.

In the vertebrate system the down-regulation of adenylate cyclase is dependent on the presence of 50-100mM NaCl (Jakobs et al., 1979). In this study 1 mM carbachol had no measurable effect on the rate of cAMP production in the absence of NaCl, in the presence of 90 mM NaCl however 1 mM carbachol reduced the adenylate cyclase activity to 60% of the basal level. This dependence on NaCl is associated in the vertebrate system with effects mediated by the GTP-binding protein N_i which is thought to mediate the down-regulation of

adenylate cyclase by several neurotransmitters (Cooper, 1981).

It has been demonstrated here that the insect CNS does, like the vertebrate, show muscarinic down-regulation of cAMP production.

-Phosphoinositide metabolism-

5.8.2

Phosphoinositide metabolism has previously been shown to be important in peripheral tissues in insects (Fain and Berridge, 1979) and in phototransduction in the photoreceptors of Limulus, another arthropod (Brown et al., 1984; Fein et al., 1984). In the insect CNS, however, although there have been reports of the component parts of the second messenger system based on PI; polyphosphoinositides (Killian and Schacht, 1979), inositoltrisphosphate (Trimmer and Berridge, 1985) and PI phosphodiesterase (Yoshioka et al. 1985) the presence of a functional, agonist-stimulated, system had not been established, before this study. The previous attempts to study the effects of neurotransmitters on the metabolism of the phosphoinositides, have found difficulty in obtaining reproducible signals. Trimmer and Berridge (1985) found evidence that there was a high basal activity of the phosphoinositide metabolic cycle. In their preparation lithium, which acts to inhibit the hydrolysis of

inositol-1-phosphate to inositol, caused greatly enhanced incorporation of exogenous [^{3}H]inositol, an effect which is observed only under stimulating conditions in other tissues (Berridge et al., 1982). They concluded that the high rate of basal turnover was obscuring any effect of the agonists that they tried. They also found that atropine, a muscarinic antagonist, was effective in reducing the production of inositol phosphates and suggested that the high basal turnover might be due to release of high levels of endogenous neurotransmitter.

The release of endogenous neurotransmitter might explain the difficulty found in this study in obtaining a measurable effect on [^{3}H]inositol incorporation by treatment with carbachol. That the eventual solution was a 1 h preincubation may also indicate that the dissection of ganglia may be so traumatic to the tissue as to cause significant release of neurotransmitter and that a long time is required for the ganglia to recover. This effect may be exacerbated by the use of metal instruments for the dissection, electrophysiologists prefer using nonconductive materials for dissection to prevent 'agony potentials' (Irving, personal communication), Ross and Brady (1986) used "glass hooks" to handle the central nerve cords in their experiments on ^{32}P labelling of phospholipids in Acheta domesticus.

This study is the first in which the insect

CNS has been shown to possess a phosphoinositide metabolism which can be stimulated by a recognized neurotransmitter agonist. The potency of the antagonists atropine and pirenzepine suggests that the receptor involved is an AChR of muscarinic pharmacology.

5.8.3

In the vertebrate CNS the mAChR is directly linked to three different effector mechanisms. Of these three, two, the down-regulation of adenylate cyclase and the enhanced metabolism of phosphoinositides, have in this programme been demonstrated to be linked to locust CNS mAChRs in an apparently similar fashion to that in the vertebrate CNS. The third, opening of potassium channels (Noma, 1986), will require an electrophysiological investigation if it is to be detected.

The down-regulation of cAMP production results in a decrease in the activity of cAMP-dependent protein kinases and the opening of potassium channels leads to hyperpolarization of the cell membrane. Thus both the down-regulation of adenylate cyclase and the opening of potassium channels are inhibitory effects in nature. Either of these mechanisms would be appropriate for the feedback inhibition of ACh release in locust synaptosomes proposed by Breer and Knipper (1984).

Breer (personal communication) has since shown that ACh release in locust synaptosomes can be enhanced

by the use of dibutyryl-cAMP. This suggests that the muscarinic autoreceptor that he postulates may exert its effect by the regulation of cAMP production. This is believed to be the case in the vertebrate where the muscarinic autoreceptor is thought to be of an M_2 pharmacology (Meyer and Otero, 1985), the mAChR subclass believed to be responsible for adenylate cyclase regulation. Breer (personal communication) has evidence that supports this theory, he finds that pirenzepine the M_1 selective antagonist inhibits the binding of [^{3}H]QNB with less potency in membranes from a synaptosomal preparation than it does for membranes which he considers to be from nerve cell bodies.

The adenylate cyclase activity in the locust has been shown to be stimulated by several amines (Morton, 1984) in both his study and those of other workers on the CNS of other insects (Combest et al., 1985; Suter, 1986 and Dudai and Zvi, 1984) octopamine has been shown to be the most effective neurotransmitter at stimulating cAMP production. It may be that ACh serves to oppose these stimulatory effects which may explain some of the difficulty found in obtaining a muscarinic effect electrophysiologically. Some electrophysiology has been done on octopaminergic systems in the insect CNS (Suter, 1986 and references therein), and an examination of the effects of specific muscarinic agonists on the amplitude of the octopamine response might prove fruitful.

The presence of a mAChR-stimulated phosphoinositide metabolism, which is stimulatory through the activation of both calcium dependent protein kinases and protein kinase C, may indicate that there should be a stimulatory mAChR response in the locust CNS. Such a response has not been identified in the CNS but the excitatory response observed by Fulton (1982) presynaptic to the neuro-muscular junction in the locust may be a candidate. The characteristics of the response that Fulton (1982) found to the specific muscarinic agonist acetyl-ß-methylcholine are consistent with a second messenger controlled process. The onset of the effect is slow, compared with the nicotine evoked effect, and lasts much longer. Furthermore Fulton (1982) presents evidence that the effect is due to a facilitation of neurotransmitter (probably glutamate) release from the motorneurone. It has since been shown that phorbol esters capable of stimulating protein kinase C, which physiologically responds to diacylglycerol produced from PIP_2 cleavage, can enhance neurotransmitter release from synaptosomes (Nichols *et al.*, 1987). So mAChR stimulation of the phosphoinositide system must be a possibe mechanism for presynaptic control of the excitatory amino-acid neuromuscular junction.

-Muscarinic receptor heterogeneity-

5.9

In vertebrate tissues the mAChR has been found to show two forms of binding heterogeneity, which are only partially linked (Hoss and Ellis, 1985). Birdsall et al. (1978, 1980) found that the binding of agonists did not fit one site models. In addition the muscarinic antagonist pirenzepine has been shown in vertebrate tissues to differentiate between at least two classes of mAChR not distinguished by the classical antagonists such as QNB and atropine (Hammer et al., 1980). It seems that these two forms of mAChR heterogeneity are not equivalent as Watson et al. (1986) have shown that there is still evidence for receptor heterogeneity when they used agonists to inhibit the binding of low concentrations of [^{3}H]pirenzepine, which would be expected to be binding to only the M_1 subclass.

The mAChR in insect nervous tissue has previously been shown to possess heterogeneity in the inhibition of [^{3}H]QNB binding by agonists. Aguilar and Lunt (1984) calculated the Hill coefficients of both acetylcholine and carbachol to be less than 0.5, Whyte and Lunt (1986) presented data on the inhibition of [^{3}H]QNB by another muscarinic agonist oxotremorine, recalculation of their data gives a Hill coefficient of 0.65 (see Figure 23 for Hill plots calculated from their data) in the absence of guanine nucleotides although

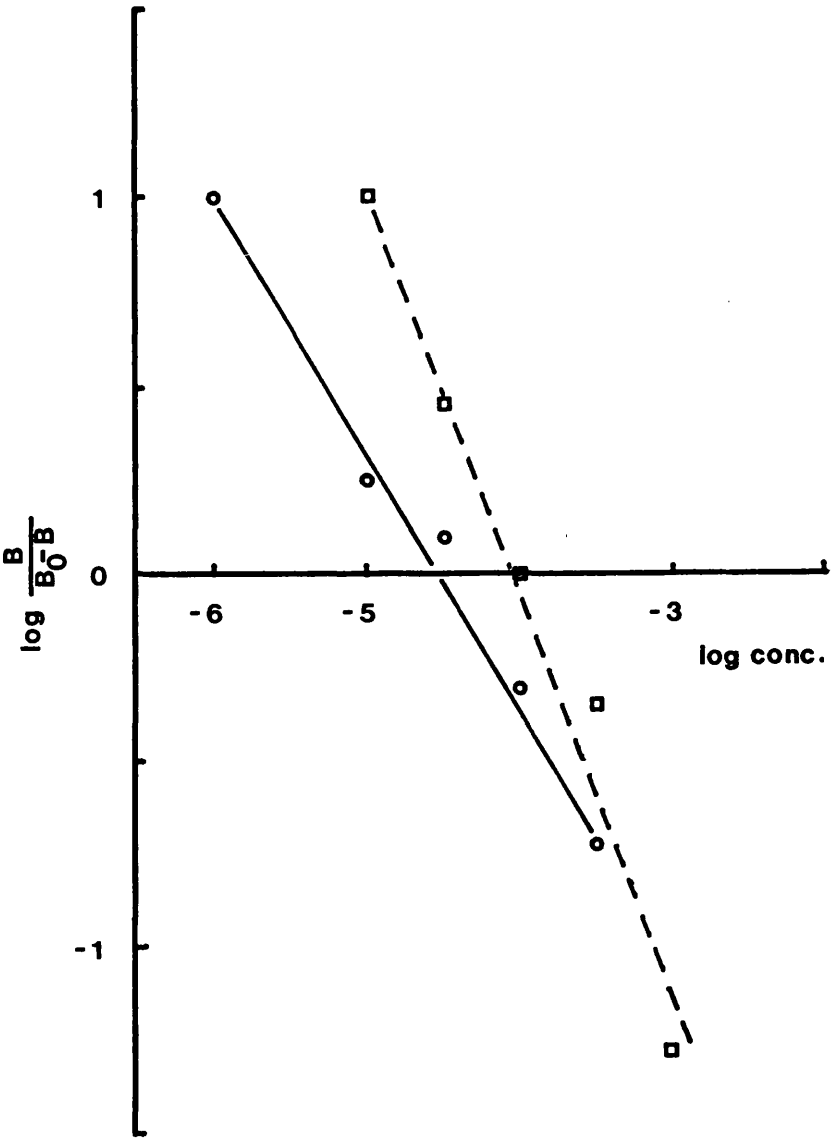

Figure 23. Hill Plot of the inhibition of [^{3}H]QNB by oxotremorine, recalculated from Whyte and Lunt (1986).

Data recalculated from the figure of Whyte and Lunt (1986), inhibition of the binding of 4 nM [^{3}H]QNB to locust ganglionic membranes in the presence (□——□) and absence (o——o) of 100 μM GTP.

Aguilar and Lunt (1984) calculated a Hill coefficient of 0.92 for the same ligand.

The subclass-selective antagonists have not previously been used in insect tissues. This study has shown that in the locust CNS pirenzepine inhibits high-affinity [^{3}H]QNB binding in a concentration dependent fashion and that the Hill Plot of the results has a slope less than unity. Computer fitting of the curve gives a better fit for a two site model than for a one site model, and thus it would appear that the mAChR in the locust CNS are not a homogenous population of binding sites.

The presence of two mAChR linked effector mechanisms in the same tissue would seem to confirm this heterogeneity of receptors as there is evidence in vertebrates that the different receptor populations, as distinguished by pirenzepine and other selective antagonists, are coupled to different effector mechanisms (Fisher, 1986). It is generally thought that in vertebrate tissues the receptors with the highest affinity for pirenzepine, called M_1 by some workers, are those linked to the activation of phosphoinositide metabolism, while the M_2 receptors are linked to the other effector mechanisms; adenylate cyclase regulation and potassium channel activation. This may also be the case in the locust as the enhancement of PI turnover produced by 1 mM carbachol is completely blocked by pirenzepine at a concentration of 5×10^{-6} M, which is a

concentration below the K_i for the low affinity site for pirenzepine determined by computer fitting to the competition curve for pirenzepine against [^{3}H]QNB.

Two other antagonists which have been reported to differentiate between three different subclasses of mAChR are AF-DX 16 (Hammer et al., 1986) and hexahydro-siladifenidol (Mutschler and Lambrecht, 1984), Birdsall et al. (in press) have used these three compounds to classify the three putative subtypes of mAChR by the rank order of potency for each. The results obtained in this study using these compounds suggest that the pharmacology in this tissue is a combination of the types described by Birdsall et al. (in press) as "Neuronal" and "Gland". This assignment can only be tentative and is based on these arguments: (a) pirenzepine is most effective at distinguishing between these subclasses in the rat and gives the lowest Hill coefficient of the three compounds in this work, whereas AF DX-116 and hexahydrosiladifenidol do not differentiate between the two subclasses as well in the rat, and have Hill coefficients above 0.6 in this study (Table 10); (b) the order of potency in this study (hexahydrosiladifenidol > pirenzepine > AF DX-116) is the same as that expected in the rat for a mixture of these two subclasses.

The recent reports by Kubo et al. (1986a,b) of the amino acid sequences of mAChRs which they think

represent two different subtypes of mAChR, suggests that the different subtypes may all be different but homologous gene products. If this proves to be so then the presence of a heterogeneous population of mAChRs in the locust would suggest that the evolutionary divergence of the different mAChRs took place before the divergence between the arthropod and the vertebrate lineages.

If this argument holds true then it would be interesting to follow the comparative pharmacology of mAChR back through phyla with lineages which are thought to have diverged earlier still and perhaps discover something of the origin of the molecular complexity of the higher nervous systems. This approach may also be applicable to other receptor systems, where it is possible, by the careful choice of selective ligands to distinguish between subtypes of a receptor which share an extensive common pharmacology. For example the different subtypes of the mAChR share an extensive common pharmacology with respect to atropine, QNB, acetyl-β-methylcholine etc., whereas nAChR and mAChR share only a limited pharmacology i.e. ACh and carbachol and are not directly related.

On a different scale the coexistence of receptors of completely different pharmacologies may give evidence for molecular evolution. This follows the evidence from molecular genetics studies that the mAChR is homologous in both DNA and protein sequence with the

β-adrenergic receptor, and the hypothesis that they may have evolved from a common ancestor, along with a protein as functionally distinct as rhodopsin (Kubo et al., 1986a,b; Birdsall and Hulme 1986).

This evidence may be used to propose a system for receptor classification which is based on information from molecular genetics which may correlate with the basic method of signal transduction. Studies on the comparative pharmacology of receptors in different phyla may, thus, be of great use if applied in conjunction with molecular biology, gene cloning and sequencing, in unravelling the course of the molecular evolution of the nervous system.

-Further Work-

4.11

Although the presence of more than one subtype of mAChR in the locust has been shown the precise relationship between them and the subtypes of mAChR which have been characterised in the vertebrates has not been firmly established. This would require extending the range of subtype-selective compounds studied both in binding assays and in the control of second messenger systems.

In particular the use of [^{3}H]pirenzepine which is now commercially available would allow

pharmacological characterization of the subset of receptors with a high affinity for pirenzepine, this technique has been used in rat brain by Birdsall *et al.* (in press). It should be possible to establish the pharmacology of the down-regulation of cAMP production, although the precise pharmacology of the phosphoinositide response may require that the assay system be further optimised.

If evidence from binding studies was obtained for more than two subtypes of mAChR then electrophysiological studies could be designed to search for a mAChR-linked potassium channel similar to that found in vertebrates.

To confirm the pharmacological identification of different classes of mAChR in the locust will probably require the application of the techniques of molecular genetics using as probes sequences obtained from the genes for the mAChR from vertebrates by Kubo *et al.* (1986a,b) perhaps concentrating on those regions which are conserved in the mAChR and the β-adrenergic receptor.

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